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August 18, 2008

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Re: US Patent Appl. Serial No.: 10/026,931
Inventors: Mahler et al.
For: Allergy Vaccines
Attorney Docket No.: 966927.00007

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office:

1. Appeal Brief (39 pages; 2-Tab Appendix);
2. Check No. in the amount of $255.00;
3. One (1) return postcard

It is respectfully requested that the attached postcard be stamped with the date of filing of these documents, and that it be returned to our courier.

Respectfully submitted,
Dobe Law Group, LLC

Christopher E. Aniedobe, Esq.
Reg. No. 48,293

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Mahler et al	
Serial No.: 10/026,931	
Attorney Docket No.: 966927.00007	*Allergy Vaccines*
Filing Date: December 27, 2001	
Examiner: Szperka, Michael Edward	
Art Unit: 1644	

APPEAL BRIEF

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The above-identified patent application comes before the United States Patent and Trademark Office Board of Appeals and Interferences from the Final Rejection of Claims 33, 34, 37-41, 43-46, 48-50, and 52 -60 by the Examiner in an Official Action mailed December 17, 2007. Pursuant to the Notice of Appeal filed June 16, 2008, set forth below is the Appellant's Brief. A Check in the amount of $255.00 is herewith submitted for payment of the fee under 37 C.F.R. §41.20(b)(2).

Appeal brief being due on or before Monday, August 18, 2008, it is not believed that any extension of time is necessary. The Commissioner is hereby authorized to charge any fees which may be required during the entire pendency of the appeal, or credit any overpayment, to Deposit Account 50-4336.

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I. Real Party in Interest:

The real party in interest in the above-captioned application is BIOMAY AG. ("Appellant"), a corporation of the Country of Austria, and having a place of business at Lazarettgasse 19, 1090 Vienna, Austria. The application has been assigned of record to SHAN-Beteiligungsgesellschaft mbH by the inventors: Susanne Vrtala, Roland Suck, Oliver Cromwell, Helmut Fiebig, Dietrich Kraft, and Rudolf Valenta and by Vera Mahler to Biomay AG. The original Assignment was recorded at the United States Patent and Trademark Office, Assignment Division, on Reel 013080 and Frame 0382 on the July 10, 2002, to SHAN-BETEILIGUNGSGESELLSCHAFT M.B.H.; which later merged with Biomay Produktions und-Handels-Aktiengesellschaft as recorded on Reel 021205 and Frame 0921 on June 4, 2008; which later changed its name to BIOMAY AG as recorded on Reel 021085 and Frame 0018 on June 4, 2008.

II. Related Appeals and Interferences:

Appellant has filed an Appeal brief before the Board of Patent Appeals and Interferences in U.S. Patent Application No. 10/026,914 entitled, "*Allergy Vaccines Containing Hybrid Polypeptides.*" There are no other appeals or interferences known to Appellant or Appellant's legal representative which will directly affect or be directly affected by or have a bearing on the Board's decision in this present appeal.

III. Status of Claims:

Claims 24-34, 37 – 41, 43 – 46, 48 – 50 and 52 – 60 are pending in this Application. Claims 24 – 32 were withdrawn, and claims 1-23, 35, 36, 42, 47, and 51 were cancelled.

Claims 33, 34, 37-41, 43-46 and 48-54 were finally rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement, in an Office Action mailed December 17, 2007.

Claims 33, 34, 37-41, 43-46 and 48-54 were finally rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement, in an Office Action mailed December 17, 2007.

Claims 33, 34, 37-40, 43-46, 48, 49, 51, 53 and 54 were finally rejected under 35 U.S.C. § 102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Vrtala et al. (J. Immunol., December 1, 2000, 165:6653-6659), in an Office Action mailed December 17, 2007.

Claims 33, 34, 37, 46, and 48-54 were finally rejected and new claims 55-60 were also rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Valenta et al. (WO 99/16467) as evidenced by Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659) in an Office Action mailed December 17, 2007.

Claims 33, 49, and 50 were finally rejected and new claims 55, 56, 57, 59, and 60 were rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659) in view of Hem et al. (Chapter 9 of Vaccine Design: The Subunit and Adjuvant Approach, 1995, pages 249-76) in an Office Action mailed December 17, 2007.

Claims 33, 38, and 41 were finally rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659) in an Office Action mailed December 17, 2007.

Claims 33, 38 – 41, and 43 – 45 were finally rejected and new claim 56 was rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Valenta et al. (WO 99/16467) in an Office Action mailed December 17, 2007.

. Claims 33, 34 37- 41, 43 – 46, 48 – 50, 52 – 60 are the subject of the present appeal.

IV. Status of Amendments:

No amendments have been filed subsequent to the Final Rejection. All amendments have been entered and are reflected in the claims appendix.

V. Summary of Claimed Subject Matter:

As recited in independent claim 33, the present invention is directed to a method of treating or preventing a human IgE-mediated allergic disorder resulting from exposure to the major allergens of alder, hazel and birch, comprising periodically administering for a number of times to a patient in need thereof, a composition comprising one or more immunotherapeutic agents derived from Bet v 1, which induce IgE-blocking antibodies and wherein the allergenic activity of the derivative is 50% or less compared to the allergenic activity of naturally occurring Bet v 1 allergen. See Example 2, Page 9.

Claims 34 and 37 depend from claim 33 and further limit the allergenic activity of the derivative as compared to the allergenic activity of naturally occurring Bet v 1 allergen.

Claims 38 depend from claim 33 and further limit the period between administrations of the immunotherapeutic composition to at least 14 days.

Claims 39 and 40 depend from claim 33 and further limit the number of times the immunotherapeutic composition is administered.

Claim 41 depends from claim 38 and relates to the length of the time intervals between the third and the fourth administration of the immunotherapeutic composition.

Claims 43 to 45 depend from claim 33 and relate to the dosages of the immunotherapeutic composition.

Claim 46 depends from claim 33 and relates to Bet v 1 as the naturally occurring allergen.

Claim 48 depends from claim 33, wherein the immunotherapeutic composition. Further comprises an adjuvant.

Claims 49 and 50 depend from claim 33, and are directed to adsorbate-bound immunotherapeutic agents.

Claims 52 to 54 depend from claim 33, and are directed to particularly identified derivatives of Bet v 1.

Independent claim 55 relates to a method of treating a human IgE-mediated allergic disorder resulting from exposure to the major allergens of alder, hazel and birch, comprising periodically administering for a number of times to a patient in need thereof, a composition comprising one or more immunotherapeutic agents adsorbed on aluminum hydroxide, said agent being a fragment or oligomer of Bet v 1, said agent capable of inducing IgE-blocking antibodies and having allergenic activity which is 50% or less compared to the allergenic activity of Bet v 1. See Examples 2 – 4, pages 9 – 14.

Claims 56 – 59 depend from claim 55 and further limit the periodicity of administration or identifies specifically enumerated derivative of Bet v 1.

Independent claim 60 relates to a method of preventing a human IgE-mediated allergic disorder resulting from exposure to the major allergens of alder, hazel and birch, comprising periodically administering for a number of times to a patient in need thereof, a composition comprising one or more immunotherapeutic agents, adsorbed on aluminium hydroxide, said agent selected from the group consisting of amino acid sequence 1-73, amino acid sequence 74 – 159, and oligomers of Bet v.1. See Examples 2 – 4, pages 9 – 14.

Prior to the claimed invention, allergen-specific immunotherapy was based on systemic administration of crude, ill-defined, non-standardizeable allergen extracts to induce allergen-specific unresponsiveness at a great risk of anaphylactic side effects and without regards to the specific sensitization profile of a given patient. The claimed invention, among other things, has overcome the problems of the prior art related to the use of non-standardizeable crude allergen extracts by using recombinant allergens of known composition which induce blocking antibodies and thereby reduce the number of administrations and increase the time intervals between

administration. Candidate immunotherapeutic agents have been limited to derivates or oligomers of Bet v 1 which induce IgE-blocking antibodies and have allergenic activity that is at most 50% compared to the allergenic activity of naturally occurring Bet v.1 allergen.

VI. Grounds of Rejection to be Reviewed:

Issue 1

Whether claims 33, 34, 37-41, 43-46 and 48-54, comply with the enablement requirement under 35 U.S.C. §112, first paragraph.

Issue 2

Whether claims 33, 34, 37-41, 43-46 and 48-54, comply with the written description requirement under 35 U.S.C. §112, first paragraph.

Issue 3

Whether claims 33, 34, 37-40, 43-46, 48, 49, 51, 53 and 54 are anticipated under 35 U.S.C. § 102(b) or, in the alternative, unpatentable under 35 U.S.C. § 103(a) over Vrtala et al. (J. Immunol., December 1, 2000, 165:6653-6659).

Issue 4

Whether claims 33, 34, 37, 46, and 48-54, 55-60 are anticipated under 35 U.S.C. § 102(b) by Valenta et al. (WO 99/16467) as evidenced by Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659).

Issue 5

Whether claims 33, 49, 50, 55, 56, 57, 59, and 60 are unpatentable under 35 U.S.C. § 103(a), over Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659) in view of Hem et al. (Chapter 9 of Vaccine Design: The Subunit and Adjuvant Approach, 1995, pages 249-76).

Issue 6

Whether claims 33, 38, and 41 are unpatentable under 35 U.S.C. § 103(a), over Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659).

Issue 7

Whether claims 33, 38 – 41, 43 – 45, and 56, are unpatentable under 35 U.S.C. § 103(a), over Valenta et al. (WO 99/16467).

VII. Grouping of Claims:

There are three groups of claims, which stand or fall separately. Group 1 consists of independent claim 33 and dependent claims 34, 37, 38, 39, 40, 41, 43, 44, 45, 46. 48, 49, 50, 52, 53 and 54.. Group 2 consists of independent claim 55 and dependent claims 56 - 59. Group 3 consists of independent claim 60.

VIII. Argument:

Issue 1

Whether claims 33, 34, 37-41, 43-46 and 48-54, comply with the enablement requirement under 35 U.S.C. §112, first paragraph.

The Examiner admitted that although the specification enabled methods of treating birch allergy by administering trimers of Bet v 1, administering amino acid fragment 1-73 of Bet v 1, or amino acid fragment 74-259 of Bet v 1, that the specification did not enable treatment or prevention of alder, birch,, and hazel allergy by administering generic derivatives of the major allergens of alder, birch, and hazel. See Dec. 17, 2007, Final Office Action, page 3.

The Examiner asserted that "Applicants' claimed invention recited methods of treating or preventing an IgE-mediated allergic disorder, but the specification does not appear to define the term prevent. Allergic reactions occur subsequent to allergen re-exposure, and as such one reasonable interpretation of "prevention" is that therapy needs to be initiated prior to re-exposure to the allergen such that the development of an allergen specific IgE response never occurs. Another reasonable interpretation of "preventing" is that the therapeutic method is 100% effective in 100% of patients." See April 20, 2007, Office Action page 4.

The Examiner appears to take further issue with the cross-reactivity of the immunotherapeutic agents of the present invention stating that "the allergic epitopes which mediate allergy to alder, birch, and hazel pollen are not coextensive such that administration of a derivative of any one allergen of one tree pollen would necessarily be effectively cross react with all major allergens of alder, birch, and hazel." See April 20, 2007, Office Action page 7.

The Examiner appears to take further issue with the breadth of the claims stating that the independent claim recites a method wherein an agent is administered to a patient, wherein the agent is identified via a screening assay that selects agents which comprise the functional properties of inducing an IgE-blocking antibody response and comprise reduced allergenic activity as compared to the native allergen See April 20, 2007, Office Action page 4.

For primarily the above reasons, the Examiner concluded that specification did not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate with its scope.

a. The Examiner's Construction of "Prevent" is Scientifically Untenable and therefore per se Unreasonable

In the April 20, 2007, Office Action, the Examiner stated that "Applicant's claimed invention recites methods of treating or preventing an IgE-mediated allergic disorder, but the specification does not appear to define the term prevent. Allergic reactions occur subsequent to allergen re-exposure, and as such one reasonable interpretation of "prevention" is that therapy needs to be initiated prior to re-exposure to the allergen such that the development of an allergen specific IgE response never occurs. Another reasonable interpretation of "preventing" is that the therapeutic method is 100% effective in 100% of patients." See April 20, 2007, Office Action page 4, ¶ 3.

Although the Examiner is required to accord the elements of a claim their broadest reasonable interpretation, it is per se unreasonable to accord an interpretation that is scientifically untenable. Applicants had not defined the term "prevent" and see no reason to

where the plain and ordinary meaning of that term, within the context of the invention, is not inconsistent with its usage.

According to the Examiner, the term, "prevent' lends itself to two reasonable interpretations. In one interpretation, the Examiner believes that prevent may be construed to mean that "the therapy needs to be initiated prior to re-exposure to the allergen such that the development of an allergen specific IgE response never occurs." The other "reasonable interpretation" of "prevention" according to the Examiner is that the therapeutic method is 100% effective in 100% of patients.

It is clearly erroneous for the Examiner to apply the "100% effectiveness in 100%" of patients as an interpretation of "prevent" when there is no such thing as a therapy that is 100% effective in 100% of patients. Because it is an untenable proposition, it is per se unreasonable.

Within the context of the invention, there are two at risk people for whom this therapy would generally have patentable utility. The first at risk group are those who have been pre-sensitized by prior exposure to the wild type allergen and as would be treated by administering the compositions of the present invention. The other at risk group are whose who have not been sensitized by prior expose but whose allergic disorder can be prevented by administering the composition of the present invention in order to block sensitization. For instance, it is well known that children from allergic parents have a much greater risk to develop allergies. Also, those living proximal to the geographic distribution of the trees are also an at risk group whose sensitization can be blocked by treatment with the compositions of the present invention. Applicants do not see any contextual ambiguity with respect to the use of the word "prevent" and respectfully ask the Examiner to withdraw this ground for rejection.

b. The Cross-Reactivity of Derivatives of Bet v 1 with Alder and Hazel Allergens is a Settled Proposition and it is Reasonable to expect that and derivative of Bet v 1 which induce blocking antibodies will comprise substantial cross reactivity

With respect to the cross-reactivity of the immunotherapeutic agents of the present invention, the Examiner stated that the claims at issue are not enabled since "cross-reactivity is not guaranteed" December 17, 2007, Office Action, page 6, ¶ 3.

As pointed out in Applicant's October 19, 2007, Response and Amendments, as far back as 1989, Niederberger et al. (J. Allergy Clin Immunol 1998; 102:579 – 91 (cited as Reference 25 in Mahler et al. (2004) Clin. Exp. Allergy 2004: 34:115-122; observed that recombinant birch pollen allergens contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen. Additionally, Mahler et al. (2004), Id., showed that genetically engineered rBet v 1 derivatives induced IgG1 and IgG2a/b antibodies in mice which cross-reacted with natural Bet v1, as well as Bet v 1- related allergens from alder and hazel and blocked allergic patients' IgE binding to Bet v 1. As presented, the immunotherapeutic fragments of the present invention are all derived from Bet v 1. and the cross reactivity of those fragments with alder and hazel allergens has long been a settled proposition and should thus obviates this basis for rejection.

The Examiner countered that the Niederberger et al. allergens were full length Bet v1 and Bet v2, whereas the instant claims were not necessarily limited to full length molecules and as such it is unreasonable that "even shorter sequences will comprise 100% cross-reactivity."

The Examiner's position is, however, clearly in error because to make a valid rejection, the Examiner must evaluate all the facts and evidence and state why one would not expect to be able to make the extrapolation, considered unreasonable by the Examiner, across the entire scope of the claims. MPEP 2164.02. In fact, "[T]he mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it." 822 F.2d at 1078, 3 USPQ2d at 1304 (quoting *In re Chilowsky*, 229 F.2d 457, 461, 108 USPQ 321, 325 (CCPA 1956)). As a matter of law, the specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970).

The Examiner, appears guided in error, as will be more fully developed below, to require that for the claims to be enabled, that the inventors must specifically enumerate all the derivatives of Bet v 1 and must specifically test them for 100% cross reactivity. The inventors had not claimed 100% cross-reactivity and it is in error to read limitations into the claims. Moreover, it can reasonably be predicted that a Bet v 1 derivative which induces IgE-blocking antibodies and has allergenic activity of 50% or less compared to the allergenic activity of naturally occurring Bet v 1 allergen will have the IgE epitopic profile for substantial cross-reactivity since recombinant Bet v 1 has been shown to contain most of the IgE epitopes of Birch, Hazel and Alder. In other words, if the Bet v 1 derivatives meet the blocking antibody induction test and the 50% or less allergenic activity test, it can reasonably be predicted, and thus enabled, that it would have substantial cross-reactivity.

c. The Claimed Methodology is Heuristic and the Examiner Failed to Appreciate the Fundamental Nature of the Claims

The Examiner asks: How would a skilled artisan know what structures/agents/derivatives meet the recited functional limitations prior to administering the structure/agent/derivative to a patient? See Dec. 20, 2007 Office Action, page 6, ¶ 5. The sum and substance of the Examiner's arguments appear in the following paragraph:

> [T]here currently is no art recognized method to distinguish allergic from non-allergic molecules (such as derivatives comprising fragments and oligomers of allergens) on an a priori structural basis (Blumenthal et al., see particularly the last sentence of the third complete paragraph of page 39). If the identity of the IgE binding epitopes that give rise to the allergic activity of an allergen are precisely known, it is not predictable as to which amino acid positions within an epitope need to be altered by site directed mutagenesis such that IgE binding is abrogated (Burks et al., Eur. J. Biochem., 1997, 245:334-339, see entire document, particularly the top right column of page 338). Even when a precise amino acid within in the epitope to be altered is identified, the choice of what that amino acid should be mutated to by site directed mutagenesis is not predictable since some substituted amino acids reduce IgE binding while others have no effect or unexpectedly increase IgE binding (Nishiyama et al., US Patent 6,187,311, see entire document, particularly lines 4-30 of column 3, and Reese et al., J. Immunol., 2005, 175:8354-8364, see entire document, particularly the paragraph that spans pages 8357 and 8358, Table I and Figure 2). The specification does not appear to teach what changes are to be made in naturally occurring allergens to make derivatives that satisfy the recited functional criteria, and the teachings of the art indicate that the structure of the material obtained as an immunotherapeutic agent comprising reduced allergenic activity at the conclusion of a screening protocol cannot be predicted. Given the above, it appears that a skilled artisan would need to rely on trial and error to identify derivatives suitable for use in the recited method. April 20, 2007, Office Action page 6, ¶

Whereas in a different context, the Examiner's arguments would be proper, in the instant case, they are improper and betray a fundamental lack of appreciation of the heuristic nature of the invention as claimed which obviates the very same issues which the Examiner identifies as problematic in terms of enabling inventions in this area of art. .

By heuristic, Applicants believe that they invented a rapid, easily attainable, method of discovering and using Bet v 1 derivatives as immunotherapeutic agents.

By way of definition in this context, a **heuristic** is a technique designed to solve a problem that ignores whether the solution can be proven to be correct, but which usually produces a good solution or solves a simpler problem that contains or intersects with the solution of the more complex problem. A method of rapid screening for immunotherapeutic agents is in and of itself, useful, inventive, and patentable and the Examiner has failed to recognize this fundamental character of the invention.

Prior to this invention, the conventional method of desensitization of allergy patients is by the use of systemic administration of crude, nonstandardizeable allergen extracts without regards to their exact chemical composition. On the other hand, great amounts of scientific endeavors have also been put forth in the characterization of known allergens, their epitopic mapping; their primary, secondary, and tertiary structure characterization; as well as experimentally onerous substitution, addition, and deletion studies aimed at potentiating natural allergens or derivatives thereof for use as immunotherapeutic agents. It does clearly appear as if the Examiner, informed by the degree of onerousity of those experimentally intensive routes, imposes a standard of 35 U.S.C. 112, first paragraph, that is inappropriate for the claimed invention under review.

Not only have scientists wrestled with the experimentally onerous characterization and potentiation of known allergens for use as immunotherapeutic agents, there is also a need for cross-sensitization of patients across a broad class of allergens by using the least amount of standardizeable allergen derivatives. The thinking prior to the instant invention is that this can be accomplished by systemic administration of a cocktail of standardized allergen derivative.

The invention under consideration is premised on the principle that rather than making, purifying, and standardizing a cocktail of allergen derivatives, that derivatives of Bet v 1,which can all be prepared by routine methods known in the art, can be rapidly screened for use as immunotherapeutic agents. The second leg on which this heuristic methodology stands is that rather than go the experimentally undue and onerous route of molecularly characterizing and epitopically mapping, substituting, adding, deleting, and derivatizing these derivatives, that one could simply follow the far less experimentally involved step of administering said derivative to a test animal and selecting as immunotherapeutic agents those derivatives that induce IgE-blocking antibodies and have 50% or less allergenic activity compared to naturally occurring allergens. See Examples 4 and 5, pages 12 -14.

In other words, the inventors have now enabled, the equivalent of an immunological litmus test used as a heuristic; a technique designed to solve the problem of immunotherapeutic agents that ignores whether the structure of the resulting agent can be proven in the molecular sense, as advocated for by the Examiner, to have its epitopes favorably positioned as such, but which technique produces a good solution or solves a simpler problem that contains or intersects with the solution of the more complex problem. Granted, this rapid screening test involves some amount of experimentation but it is a mere routine, a mere litmus test; not one that can be remotely characterized as undue given the routes which scientists have heretofore followed. It is this failure to appreciate that this invention is in and of itself, a rapid method of discovering immunotherapeutic agents recombinantly derived from Bet v1 , that informs the Examiner's manifestly erroneous application of the enablement requirements of 35 U.S.C. 112, first paragraph.

Issue 2

Whether claims 33, 34, 37-41, 43-46 and 48-54, comply with the written description requirement under 35 U.S.C. §112, first paragraph.

Claims 33, 34, 37-41, 43-46, and 48-51 stand rejected under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner asserts that claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention for the reasons of record.

The crux of the Examiner's argument is as follows:

> [T]he instant specification does not disclose how the structure of the derivative is correlated with the functional properties of eliciting IgE-blocking antibodies and reduced allergenicity. Even if a skilled artisan could obtain derivatives using a screening assay, the structure of derivatives that comprise the desired functional properties would not be known until such time as the screening assay is completed. How can the specification provide adequate written description for a derivative that cannot be known until the completion of a screening method that has yet to be performed? Note that in Univ. of Rochester v. G.D. Searle & Co., 69 USPQ2d 1886 (Fed.Cir. 2004) the court held that the disclosure of a screening assay and the recitation of functional properties of molecules obtained upon completion of said screening assay did not provide adequate written description for the molecules themselves. More specifically, the court held that patented claims which encompassed methods of treatment with undiscovered Cox-2 inhibitors such as Celebrex were invalid for lack of possession of the genus of administered Cox-2 inhibitors.
>
> The instant case is similar in that the specification provides a screening assay and recites functional properties of allergen derivatives that are yet to be discovered. Further, the three Bet v 1 derivatives that are disclosed (trimer, amino acids 1-73 and amino acids 74-159) are not representative species of the genus of all major allergens of alder, hazel, and birch. See April 20, 2007, Office Action. Page 11, ¶ 2, 3.

Applicants traverse this rejection because the method of treating and/or preventing human IgE-mediated disorders as a result of exposure to the major pollens of alder, birch and

hazel by periodic administration of a composition comprising derivatives of Bet v 1 capable of inducing blocking antibodies and having allergenic activity of 50% or less compared to naturally occurring allergens is described in the specification in such a way as to reasonably convey to one of ordinary skill in the art at the time the application was filed that the Applicant had possession of the claimed invention as required by 35 U.S.C. §112, first paragraph.

35 U.S.C. §112, first paragraph sets forth in part:

> the specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The MPEP summarizes the Federal Circuit's characterization of the written description requirement by stating that, "[a]n applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention." MPEP §2163 *citing Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572 (Fed. Cir. 1997). Further, the MPEP states that "[p]ossession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was 'ready for patenting' such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention." MPEP §2163 *citing Pfaff v. Wells Electronics, Inc.*, 525 U.S. 55, 68 (1998); *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, (Fed. Cir. 1997), *cert. denied*, 523 U.S. 1089 (1998); and *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206 (Fed. Cir. 1991).

a. The Examiner errs by requiring a correlation of the functional properties with structural characterization of the derivatives of Bet v 1, where as here, the invention could be practiced without necessarily knowing the molecular structure of the derivatives.

The Examiner wonders how the specification can provide adequate written description for a derivative that cannot be known until the completion of a screening method that has yet to be performed citing that in Univ. of Rochester v. G.D. Searle & Co., 69 USPQ2d 1886 (Fed.Cir. 2004) the court held that the disclosure of a screening assay and the recitation of functional properties of molecules obtained upon completion of said screening assay did not provide adequate written description for the molecules themselves.

The instant case, however, differs from Univ. of Rochester v. G.D. Searle & Co., 69 USPQ2d 1886 (Fed.Cir. 2004) in the sense that the molecular structure of the therapeutic agent does not need to be known in order to treat or prevent IgE mediated disorders of hazel, birch, and alder.

The Examiner has unnecessarily backed himself into an ontological inquiry that forces the misapplication of the Written Description Requirement. As is generally well known, allergen extracts continue to be used as immunotherapeutic agents without necessarily knowing the exact structural characterization of its components. Likewise, an artisan using any of the well-known derivation protocols can derivatize Bet v 1, routinely screen the derivative as stipulated in the claims and if it meets the criteria, use it in a batch-like manner without ever knowing the exact chemical structure of the derivative.

The artisan can optionally seek to further characterize the derivative but in no way, however, has failure to elucidate the structure of the derivative impaired the applicability of the invention.

b. The Claimed Methodology is Inherently Self-Validating and the Methodology does not Stand nor Fail on the Exact Molecularity of the Hybrid Polypeptides or Derivatives Thereof

If the Applicants were claiming a product, then adequate inquiry must be had as to whether they had the claimed product in their possession as at the time of filing of the Application. On the other hand, if the Applicants are claiming a method, then the inquiry as to whether they had possession of the method as at the filing of the Application need not exceed the metes and bounds of the claimed method; especially where as here, those metes and bounds have been clearly and concisely delineated.

Applicants are not claiming to be the first to make derivatives of Bet v 1, which is so routinely done and well known to one of skill in the art be used to burden the Specification. The obligation to concisely state what they have invented, invites the obligation to exclude materials which are quite trite while giving notice to one of skill in the art as to exactly what they had possession of.

In regards, therefore, to the heuristic nature of the claimed invention, it is clear and manifest error to insist that guidance as to molecularity of species amenable to the methodology, other than the restrictions appearing on the face of the invention, be provided, where as here, the invention itself is self-validating. In other words, **however derived**, and whatever the molecularity, be it a two amino-acid sequence or five-hundred amino acid sequence, a Bet v 1 derivative can now, by virtue of this invention, be for the first time, **routinely tested** as candidate immunotherapeutic agents by administering said polypeptide to a test animal and selecting as immunotherapeutic agents those fusion polypeptides that induce IgE-blocking antibodies and has 50% or less reduced allergenicity compared to wild type

allergens. By so teaching, it is manifest error to require *a priori*, that the molecularity of the chemical species amenable to the claimed methodology be the appropriate subject of the first inquiry where as here the steps in the process inherently validate and thus obviate the need for said first inquiry by means of the equivalence of a mere immunological litmus test.

The Patent and Trademark Office provides examiners with a set of guidelines to follow when examining patent applications for compliance with the written description requirement called the "Revised Interim Written Description Guidelines Training Material" ("Guidelines"). *See* 66 Fed. Reg. 1099, 1099-1111 (January 5, 2001). The Guidelines state that "[t]here is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed." 66 Fed. Reg. 1099, 1105. Further, the Guidelines state that the, "[w]ritten description for a claimed genus may be satisfied . . . by disclosure of relevant, identifying characteristics, i.e. structure or other physical and/or chemical properties . . . or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus." *Id.* at 1106. The Guidelines also provide that, "[t]he absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. [§]112, [paragraph] 1, for lack of adequate written description." 66 Fed. Reg. 1099, 1105.

On the basis of the foregoing, particularly the Examiner's failure to properly characterize the invention and the clearly erroneous misapplication of 35 USC 112, first paragraph, this ground for rejection should be removed.

Issue 3

Whether claims 33, 34, 37-40, 43-46, 48, 49, 51, 53 and 54 are anticipated under 35 U.S.C. § 102(b) or, in the alternative, unpatentable under 35 U.S.C. § 103(a) over Vrtala et al. (J. Immunol., December 1, 2000, 165:6653-6659).

Claims 33, 34, 37-40, 43-46, 48, 49, 51, 53 and 54 stand rejected under 35 U.S.C. 102(b) as anticipated by or, in the alternative, under 35 U.S.C. 103(a) as obvious over Vrtala et al. (J. Immunol., December 1, 2000, 165:6653-6659).

According to the Examiner, "Vrtala et al. teach methods of specific immunotherapy to treat birch allergy by administering derivatives of Bet v 1 (see entire document, particularly the abstract and the left column of page 6658). The administered derivatives induced the production of antibodies which precluded the binding of IgE antibodies to native Bet v 1 allergen (see particularly the abstract and Tables II-IV). The derivatives of Bet v 1 are taught as being repeatedly administered at the same concentration at intervals greater than 14 days, with such administrations occurring four times (see particularly the second and third paragraphs of the right column of page 6654). Administered dosages are taught as comprising 5μg and 20014 of Bet v 1 derivatives (ibid.). These dosages are further taught as being adsorbed onto an adjuvant (ibid. and the last sentence of the first full paragraph of the left column of page 6658)."

The Examiner appears to be driven by impermissible hindsight to misconstrue the teachings of the prior art in order to manufacture a clearly erroneous basis for obviousness.

35 U.S.C. §103(a) sets forth in part:

> [a] patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject mater as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains.

Section 103 forbids issuance of a patent when "the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have

been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." KSR Int'l Co. v. Teleflex Inc., 127 S.Ct. 1727, 1734, 82 USPQ2d 1385, 1391 (2007).

The question of obviousness is resolved on the basis of underlying factual determinations including (1) the scope and content of the prior art, (2) any differences between the claimed subject matter and the prior art, (3) the level of skill in the art, and (4) where in evidence, so-called secondary considerations. Graham v. John Deere Co., 383 U.S 1, 17-18, 148 USPQ 459, 467 (1966). See also KSR, 127 S.Ct. at 1734, 82 USPQ2d at 1391 ("While the sequence of these questions might be reordered in any particular case, the [Graham] factors continue to define the inquiry that controls.") The Court in Graham further noted that evidence of secondary considerations, such as commercial success, long felt but unsolved needs, failure of others, etc., "might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." 383 U.S. at 18, 148 USPQ at 467.

In KSR, the Supreme Court emphasized "the need for caution in granting a patent based on the combination of elements found in the prior art," *Id.* at 1739, 82 USPQ2d at 1395, and discussed circumstances in which a patent might be determined to be obvious without an explicit application of the teaching, suggestion, motivation test. In particular, the Supreme Court emphasized that "the principles laid down in Graham reaffirmed the 'functional approach' of Hotchkiss, 11 How. 248." KSR, 127 S.Ct. at 1739, 82 USPQ2d at 1395 (citing Graham v. John Deere Co., 383 U.S. 1, 12 (1966) (emphasis added)), and reaffirmed principles based on its precedent that "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results." Id. The Court explained:

> When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, §103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill.

Id. at 1740, 82 USPQ2d at 1396. The operative question in this "functional approach" is thus "whether the improvement is more than the predictable use of prior art elements according to their established functions." *Id.*

The Court explained, "[o]ften, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue." *Id.* at 1740-41, 82 USPQ2d at 1396. The Court noted that "[t]o facilitate review, this analysis should be made explicit." *Id.*, citing In re Kahn, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006) ("[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness"). However, "the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *Id.*

a) By using impermissible hindsight, the Examiner improperly failed to give due consideration to patentable differences between the cited prior art and the invention.

Coming from the same laboratory, at best the cited prior art is inchoate and contain nuggets that coincide with the invention as claimed and aided by hindsight should not preclude due consideration of the patentable differences between the invention and the prior art. The following patentable differences are evident:

1. Vrtala et al. (2000) only uses specifically identified Bet v 1 fragments and thus fails to demonstrate the general principles of the immunotherapeutic utility of a broad range of Bet v 1 allergens including fragments AND oligomers.

2. Vrtala et al. (2000) dealt with only the isolated fragments and not the fragment mix for vaccination;

3. Vrtala et al. (2000) uses mainly complete and incomplete Freunds Adjuvant (CFA,

ICFA) which is not allowed for human use and it has been recently demonstrated that hypoallergens given with CFA fail to induce allergen-specific IgG when adsorbed to Alum (See Vrtala et al. (2007) J. Immunol. 179:1730-1739. This also puts paid to the Examiner's argument that it would have been obvious to merely substitute Alum with CFA.

4. The recited dosage levels and the periodicity schedule are not subject to routine optimization. On the contrary, it is experimentally intensive and critical to successful immunotherapy.

For at least these differences, there is no basis for a finding of obviousness.

Issue 4

Whether claims 33, 34, 37, 46, and 48-54, 55-60 are anticipated under 35 U.S.C. § 102(b) by Valenta et al. (WO 99/16467) as evidenced by Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659).

The foregoing arguments are incorporated herein by reference. For at least the patentable differences between Vrtala et al. and the instant invention, there is no basis for this ground for rejection. Specifically, the Valenta reference refers to the hyposensitization of patients using in general, polymeric forms of hypo-allergenic fragments of naturally occurring allergens. While Valenta et al, illustrates the principle that polymeric fragments of naturally occurring allergens can have immunotherapeutic potentials, nothing is mentioned in Valenta et al of the periodicity element of claim 33 nor was the "50% or less" hypoallergenic activity established as a therapeutic threshold.

Issue 5

Whether claims 33, 49, 50, 55, 56, 57, 59, and 60 are unpatentable under 35 U.S.C. § 103(a), over Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659) in view of Hem et al. (Chapter 9 of Vaccine Design: The Subunit and Adjuvant Approach, 1995, pages 249-76).

The foregoing arguments are incorporated herein by reference. For at least the patentable differences between Vrtala et al. and the instant invention, there is no basis for this ground for rejection.

Issue 6

Whether claims 33, 38, and 41 are unpatentable under 35 U.S.C. § 103(a), over Vrtala et al. (J. Immunol., Dec. 1, 2000, 165:6653-6659).

The foregoing arguments are incorporated herein by reference. For at least the patentable differences between Vrtala et al. and the instant invention, there is no basis for this ground for rejection.

Issue 7

Whether claims 33, 38 – 41, 43 – 45, and 56, are unpatentable under 35 U.S.C. § 103(a), over Valenta et al. (WO 99/16467).

The foregoing arguments are incorporated herein by reference. For at least the patentable differences between Vrtala et al. and the instant invention, there is no basis for this ground for rejection.

CONCLUSION

In view of the forgoing discussion, it is respectfully submitted that the Examiner's rejections of claims 24-34, 37 – 41, 43 – 46, 48 – 50 and 52 – 60 are improper and should be reversed by the Board.

Respectfully submitted,

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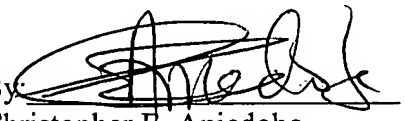

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IX. Claims Appendix

1 — 11 (canceled)

12 — 23. (canceled)

24. (withdrawn) A method of preparing a derivative from a naturally occurring allergen, wherein specific IgE binding to the derivative is 50% or less compared to the IgE binding to said naturally occurring allergen, which method comprises:
modifying said naturally occurring allergen to induce IgG antibody production wherein the production of allergen-specific IgE is reduced.

25. (withdrawn) The method of claim 24, wherein the IgE binding to the derivative is 25% or less compared to the IgE binding to said naturally occurring allergen.

26. (withdrawn) The method of claim 24, wherein the IgE binding to the derivative is 10% or less compared to the IgE binding to said naturally occurring allergen.

27. (withdrawn) The method of claim 24, wherein the IgE binding to the derivative is 5% or less compared to the IgE binding to said naturally occurring allergen.

28. (withdrawn) The method of claim 24, wherein the IgE binding to the derivative is eliminated compared to the IgE binding to said naturally occurring allergen.

29. (withdrawn) The method of claim 24, wherein the specific IgG isotype induced is IgG_1 and IgG_2.

30. (withdrawn) The method of claim 24, wherein, the specific IgG isotype induced is IgG_1,

IgG_2 and IgG_4.

31. (withdrawn) The method of claim 24, wherein the naturally occurring allergen is Bet v 1.

32. (withdrawn) The method of claim 24, wherein the naturally occurring allergen is selected from the group consisting of major grass pollen allergens, mite allergens, bee venom allergens and animal hair dander allergens.

33. (currently amended) A method of treating or preventing a human IgE-mediated allergic disorder resulting from exposure to the major allergens of alder, hazel and birch, comprising periodically administering for a number of times to a patient in need thereof, a composition comprising one or more immunotherapeutic agents derived from Bet v 1, ~~selected by a process involving:~~

~~providing derivatives from naturally occurring allergens selected from the group consisting of the major allergens of alder, hazel and birch;~~

~~challenging an immunological model with said derivatives;~~

~~selecting as immunotherapeutic agents, those derivatives~~ which induce IgE-blocking antibodies and wherein the allergenic activity of the derivative is 50% or less compared to the allergenic activity of ~~said~~ naturally occurring Bet v 1 allergen.

34. (previously presented) The method of claim 33, wherein the allergenic activity of the derivative is 25% or less compared to the allergenic activity of said naturally occurring allergen from which it is derived.

35. (canceled)

36. (canceled)

37. (previously presented) The method of claim 33, wherein the derivative elicits substantially no allergenic activity compared to the allergenic activity of said naturally occurring allergen.

38. (previously presented) The method of claim 33, wherein the period between administrations of the composition is at least 14 days.

39. (previously presented) The method of claim 38, wherein the composition is administered to the patient from three to five times.

40. (previously presented). The method of claim 39, wherein the composition is administered to the patient four times.

41. (currently amended) The method of claim 38, wherein the time interval between the third and the fourth administration ~~being longer~~ is longer than the time intervals between the first three administrations.

42. (canceled)

43. (previously presented) The method of claim 33, wherein, during each administration, substantially the same dose of the derivative is administered.

44. (previously presented) The method of claim 43, wherein, during each administration, a dose of at least 5 μg of the derivative is administered.

45. (previously presented) The method of claim 43, wherein, during each administration, a dose of at least 10 ug of the derivative is administered.

46. (previously presented) The method of claim 33, wherein the naturally occurring allergen is Bet v 1.

47. (canceled)

48. (previously presented) The method of claim 33, wherein the composition further comprises an adjuvant.

49. (previously presented) The method of claim 33, wherein the immunotherapeutic agents are adsorbed unto a pharmaceutically acceptable adsorbate.

50. (previously presented) The method of claim 49, wherein the adsorbate is aluminum hydroxide.

51. (canceled)

52. (previously presented) The method of claim 33, wherein the immunotherapeutic agent is a trimer of Bet v 1.

53. (previously presented) The method of claim 33, wherein the immunotherapeutic agent is a polypeptide consisting of amino acids 1-73 of Bet v 1 .

54. (previously presented) The method of claim 33, wherein the immunotherapeutic agent is a

polypeptide consisting of amino acids 74-159 of Bet v l.

55. (new) A method of treating a human IgE-mediated allergic disorder resulting from exposure to the major allergens of alder, hazel and birch, comprising periodically administering for a number of times to a patient in need thereof, a composition comprising one or more immunotherapeutic agents adsorbed on aluminum hydroxide, said agent being a fragment or oligomer of Bet v.1, said agent capable of inducing IgE-blocking antibodies and having allergenic activity which is 50% or less compared to the allergenic activity of Bet v. 1.

56. (new) The method of claim 55, wherein the composition is administered four times and the period between administrations of the composition is at least 14 days, and the time interval between the third and fourth administration is longer than the time intervals between the first three administrations.

57. (new) The method of claim 55, wherein the fragment is at least one selected from the group consisting of amino acid sequence 1-73 and amino acid sequence 74 – 159 of Bet v 1.

58. (new) The method of claim 55, wherein the oligomer is a trimer of Bet v 1.

59. (new) The method of claim 56, wherein the fragment is at least one selected from the group consisting of amino acid sequence 1-73 and amino acid sequence 74 – 159 of Bet v 1.

60. (new) A method of preventing a human IgE-mediated allergic disorder resulting from exposure to the major allergens of alder, hazel and birch, comprising periodically administering for a number of times to a patient in need thereof, a composition comprising one or more immunotherapeutic agents,

adsorbed on aluminium hydroxide, said agent selected from the group consisting of amino acid sequence 1-73, amino acid sequence 74 – 159, and oligomers of Bet v 1.

X. Evidence Appendix

Tab 1 Vrtala et al. (J. Immunol., December 1, 2000, 165:6653-6659) and was entered in the record by the Examiner on June 20, 2006.

Tab 2. Valenta et al. (WO 99/16467)) and was entered in the record by the Examiner on June 20, 2006.

XI. Related Proceedings Appendix

None.

T Cell Epitope-Containing Hypoallergenic Recombinant Fragments of the Major Birch Pollen Allergen, Bet v 1, Induce Blocking Antibodies[1]

Susanne Vrtala,* Cezmi A. Akdis,† Ferah Budak,† Mübeccel Akdis,† Kurt Blaser,† Dietrich Kraft,* and Rudolf Valenta[2]*

Allergen-specific immunotherapy represents one of the few curative approaches toward type I allergy. Up to 25% of allergic patients are sensitized against the major birch pollen allergen, Bet v 1. By genetic engineering we produced two recombinant (r) Bet v 1 fragments comprising aa 1–74 and aa 75–160 of Bet v 1, which, due to a loss of their native-like fold, failed to bind IgE Abs and had reduced allergenic activity. Here we show that both fragments covering the full Bet v 1 sequence induced human lymphoproliferative responses similar to rBet v 1 wild type. The C-terminal rBet v 1 fragment induced higher lymphoproliferative responses than the N-terminal fragment and represented a Th1-stimulating segment with high IFN-γ production, whereas the N-terminal fragment induced higher IL-4, IL-5, and IL-13 secretion. Immunization of mice and rabbits with rBet v 1 fragments induced IgG Abs, which cross-reacted with complete Bet v 1 and Bet v 1-related plant allergens and strongly inhibited the IgE binding of allergic patients to these allergens. Thus, our results demonstrate that hypoallergenic T cell epitope-containing rBet v 1 fragments, despite lacking IgE epitopes, can induce Abs in vivo that prevent the IgE binding of allergic patients to the wild-type allergen. The overall demonstration of the immunogenic features of the hypoallergenic rBet v 1 fragments will now enable clinical studies for safer and more efficient specific immunotherapy. *The Journal of Immunology,* **2000, 165: 6653–6659.**

Type I allergy represents a genetically determined immunodisorder that affects almost 25% of the population (1). The symptoms of type I allergy (i.e., allergic rhinoconjunctivitis, asthma, atopic dermatitis) are caused by the formation of IgE Abs against harmless Ags. Cross-linking of effector cell (e.g., mast cell, basophil)-bound IgE Abs by allergens causes immediate symptoms via the release of biological mediators (e.g., histamine, leukotriens) (1–3), whereas IgE-mediated allergen presentation to T cells greatly enhances T cell activation, release of proinflammatory cytokines, and thus late reactions (4–5). Although immediate as well as late symptoms can be ameliorated by pharmacological treatment, specific immunotherapy represents a curative approach toward type I allergy (6–8). Specific immunotherapy is based on the administration of increasing doses of the disease-eliciting allergens to induce a state of unresponsiveness toward the applied allergens. Several clinical studies document the clinical efficacy of specific immunotherapy, but the mechanisms underlying immunotherapy are not precisely known (6). One major disadvantage of specific immunotherapy is that the administration of allergenic material may cause severe and life-threatening anaphylactic side effects. To prevent spreading of allergens during injection immunotherapy and thus to reduce the rate of severe systemic anaphylactic reactions, allergen extracts were adsorbed to adjuvants (9–10). Furthermore, chemically modified allergen extracts (e.g., allergoids) with low IgE binding capacity have been developed to reduce anaphylactic side effects during specific immunotherapy (11–12).

During the last several years several groups have produced recombinant allergens by recombinant DNA technology, which equal the natural allergens regarding biochemical, biological, immunological, and structural properties (13, 14). Recombinant allergens can now be used for new forms of component-resolved diagnosis, which may represent the basis for immunotherapy tailored according to the sensitization profile of the patient (15). Moreover, recombinant DNA technology, as well as synthetic peptide chemistry, is currently applied to generate allergen derivatives for immunotherapy with reduced anaphylactic side effects (16). Here we report the characterization of recombinant hypoallergenic fragments of the major birch pollen allergen, Bet v 1, as candidate molecules for specific immunotherapy. Bet v 1 is recognized by >95% of birch pollen allergic patients, and 60% of these patients react exclusively with this allergen (17, 18). The cDNA coding for Bet v 1 was isolated (19), and recombinant Bet v 1, which has immunological and biological properties comparable to natural Bet v 1 (18, 20) and shares epitopes with homologous proteins present in the pollen of trees of the order *Fagales* and in plant-derived food was produced in *Escherichia coli* (21–24). When analyzed by circular dichroism spectroscopy, the bacterially expressed rBet v 1 wild-type molecule was folded and consisted of mixed α helical and β-sheet conformation (25, 26). The three-dimensional structure of rBet v 1 was determined by x-ray crystallography and nuclear magnetic resonance analysis (27).

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0022-1767/00/$02.00

The fact that natural and recombinant Bet v 1 fragments failed to bind IgE Abs indicated that IgE epitopes of Bet v 1 belong to the conformational (discontinuous) type (26). When we engineered two rBet v 1 fragments comprising aa 1–74 and aa 75–160, we found that the fragments, despite comprising the full Bet v 1 sequence, lacked IgE binding capacity due to a loss of their fold but induced proliferation of rBet v 1-specific T cell clones (26). Moreover, rBet v 1 fragments had a >100-fold reduced capacity to induce basophil histamine release and immediate type skin reactions in birch pollen allergic patients (26, 28, 29). Both rBet v 1 fragments also failed to induce eosinophil activation and late reactions in skin blisters of birch pollen-allergic patients (30).

Here we investigated whether the hypoallergenic rBet v 1 fragments can induce proliferation in PBMC of birch pollen-allergic patients comparable to that induced by the complete rBet v 1 wild-type molecule and thus contain the relevant Bet v 1-specific T cell epitopes. We were also interested to compare the types and levels of cytokines released from PBMCs after incubation with rBet v 1 and rBet v 1 fragments. Furthermore, we investigated whether the hypoallergenic rBet v 1 fragments, despite loss of their conformational IgE epitopes, could induce IgG Abs in mice and rabbits, which recognize the folded wild-type allergen and Bet v 1-homologous allergens from other plants. Finally, we determined whether the rBet v 1 fragment-induced Abs can inhibit the recognition of complete rBet v 1 by IgE Abs from birch pollen-allergic patients. We discuss the possible advantages of using hypoallergenic T cell epitope-containing fragments of Bet v 1 for specific immunotherapy.

Materials and Methods

Patients' sera, plasmid vectors, E. coli *strains, and recombinant allergens*

Birch pollen-allergic patients were characterized by case history and skin prick testing. In addition, sera were screened for the presence of IgE Abs to birch pollen extract by radio allergosorbent test (RAST) (Pharmacia, Uppsala, Sweden) and by IgE immunoblotting as described (21).

Plasmid pET 17b and *E. coli* strain BL 21 (DE3) (F^- *ompT* r_B^- m_B^- (DE3)) were purchased from Novagen (Madison, WI). Folded rBet v 1a, which had been expressed in *E. coli* and purified as described (31), was obtained from Biomay (Linz, Austria). rBet v 1 fragments, comprising aa 1–74 and aa 75–160, were generated by PCR, using the rBet v 1a cDNA as a template, subcloned into plasmid pET-17b, expressed in *E. coli* strain BL 21 (DE3), and purified as described (26).

Natural allergen extracts

Pollen from birch (*Betula alba*), alder (*Alnus glutinosa*), hazel (*Corylus avellana*), hornbeam (*Carpinus betulus*), and oak (*Quercus alba*) were purchased from Allergon AB (Välinge, Sweden). Golden delicious apples were purchased from a local market (Vienna, Austria). Pollen extracts were prepared by homogenizing 1 g tissue in 10 ml H_2O_{dd} containing 5 mM PMSF using an ultraturrax (IKA, Heidelberg, Germany) and extracting at 4°C for 2 h. Extracts were then centrifuged for 30 min at 20,000 × *g* to remove insoluble particles. Supernatants were lyophilized and stored at −20°C until use. Apple protein extracts were obtained by homogenizing fruits in SDS-sample buffer with an ultraturrax as described (24). Extracts were boiled for 5 min and centrifuged for 20 min at 10,000 × *g* to remove insoluble materials. All protein extracts were checked for protein quantity and quality by SDS-PAGE (32) and Coomassie blue staining (Bio-Rad, Richmond, CA) (33).

T cell proliferation and measurement of cytokines

T cell experiments were performed using PBMC of Bet v 1-sensitized individuals who had not received immunotherapy. PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were suspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (Life Technologies, Grand Island, NY), and 10% heat-inactivated FCS (34, 35). PBMC (10^5 cells/well in 96-well flat-bottom plates; Costar, Cambridge, U.K.) were stimulated with different Ag doses (20, 2, and 0.2 μg/ml) of rBet v 1 monomer and rBet v 1 fragments in triplicate. Proliferative responses were measured by [^{3}H]thymidine incorporation after 5 days (34). IL-4 was measured from 24-h supernatants and IFN-γ, IL-5, IL-10, and IL-13 were measured from 5-day supernatants by sandwich ELISA (35, 36). The sensitivities of the ELISAs were as follows: IFN-γ = 10 pg/ml (mAbs and standards were obtained from Dr. S. S. Alkan, Novartis, Basel, Switzerland); IL-4 = 20 pg/ml (mAbs and standards were obtained from Dr. C. H. Heusser, Novartis); IL-5 = 50 pg/ml, IL-10 = 50 pg/ml, IL-13 = 100 pg/ml (moAbs and standards were obtained from PharMingen, San Diego, CA). Results are shown as mean ± SD. Student's *t* test for paired samples was used for statistical analysis to compare results between paired stimulation conditions. When data were analyzed with a nonparametric test (Mann Whitney *U* test) no change of statistical significance was found between the groups.

Immunization of mice and rabbits and measurement of mouse IgG subclass responses

Eight-week-old female BALB/c mice were purchased from Charles River Breeding Laboratories (Kislegg, Germany). Animals were maintained in the animal care unit of the Department of Pathophysiology of the University of Vienna according to the local guidelines for animal care. Groups of five mice each were immunized monthly either with 5 μg of purified rBet v 1, rBet v 1 aa 1–74, or rBet v 1 aa 75–160 adsorbed to 200 μl of CFA (Sigma, St. Louis, MO). Blood samples were taken shortly before each of the four immunizations and stored at −20°C until use. IgE and IgG subclass (IgG1, IgG2a, IgG2b, and IgG3) responses to complete rBet v 1 were determined by ELISA as described (37, 38).

Rabbits were immunized three times with 200 μg of purified rBet v 1, rBet v 1 aa 1–74, or rBet v 1 aa 75–160 adsorbed to CFA over a period of 3 mo (Charles River Breeding Laboratories). Rabbit sera were tested for IgG response to complete rBet v 1 by immunoblotting as described (39).

Analysis of mouse and rabbit anti-rBet v 1 aa 1–74 and anti-rBet v 1 aa 75–160 Abs for reactivity to Bet v 1 and Bet v 1-related allergens

Approximately 1 μg/cm of purified rBet v 1 or 100 μg/cm of natural tree pollen or apple extracts were separated by 14% SDS-PAGE (32) and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) (40). Nitrocellulose strips were blocked in buffer A (50 mM sodium phosphate, pH 7.5; 0.5% w/v BSA, 0.5% v/v Tween 20, 0.05% NaN_3) two times for 5 min and once for 30 min and incubated overnight at 4°C with sera from mice or rabbits diluted 1:1000 in buffer A. Nitrocellulose strips were then washed three times in buffer A. Bound mouse and rabbit Abs were detected with a ^{125}I-labeled sheep anti-mouse Ig antiserum (Amersham, Buckinghamshire, U.K.) and an ^{125}I-labeled donkey anti-rabbit Ig antiserum (Amersham), both diluted in buffer A 1:1000, respectively, and visualized by autoradiography using Kodak XOMAT films and intensifying screens (Kodak, Heidelberg, Germany).

Inhibition of allergic patients IgE binding to rBet v 1 by anti-rBet v 1- or anti-rBet v 1 fragment-specific Abs as determined by ELISA competition

ELISA plates (Greiner, Kremsmünster, Austria) were coated with 1 μg/ml purified rBet v 1 or 20 μg/ml of pollen protein extracts overnight at 4°C. Plates were washed two times with PBS, 0.05% v/v Tween 20, blocked for 3 h at room temperature with PBS, 1% w/v BSA, 0.05% v/v Tween 20, and incubated overnight at 4°C with mouse or rabbit anti-rBet v 1, anti-rBet v 1 aa 1–74, or anti-rBet v 1 aa 75–160 Abs and, for control purposes, with the corresponding preimmune sera. Mouse preimmune and immune sera were diluted 1:20, rabbit preimmune and immune sera were diluted 1:100 in PBS, 0.5% w/v BSA, and 0.05% v/v Tween 20. Plates were washed five times with PBS and 0.05% v/v Tween 20 and incubated overnight at 4°C with sera from birch pollen-allergic patients, diluted 1:5 in PBS, 0.5% w/v BSA, and 0.05% v/v Tween 20. Plates were again washed five times with PBS, 0.05% v/v Tween 20 and bound IgE was detected with alkaline phosphatase-coupled mouse monoclonal anti-human IgE Abs (PharMingen), diluted 1:1000 in PBS, 0.5% w/v BSA, 0.05% v/v Tween 20 for 1 h at 37°C and 1 h at 4°C. After washing five times with PBS and 0.05% v/v Tween 20, plates were incubated in the dark with alkaline phosphatase substrate (Sigma) and absorbance was determined with an ELISA reader (Dynatech, Denkendorf, Germany). The percentage of reduction of human IgE binding after preincubation with mouse and rabbit immune sera was determined according to the formula: % inhibition of IgE binding = $100 - OD_I/OD_P \times 100$, where OD_I and OD_P represent extinctions after preincubation with immune serum and preimmune serum, respectively.

Results

Hypoallergenic rBet v 1 fragments induce lymphoproliferative responses and cytokine release from PBMC of birch pollen-allergic patients

To investigate whether rBet v 1 fragments contain the relevant Bet v 1-specific T cell epitopes, we performed cultures for proliferation and cytokine production with PBMC of birch pollen-allergic patients. Fig. 1*A* shows that rBet v 1 fragments as well as complete rBet v 1 induced a dose-dependent proliferation of PBMC from birch pollen-allergic patients. In all of the seven experiments performed with PBMC from five birch pollen-allergic individuals, the C-terminal rBet v 1 fragment aa 75–160 induced significantly higher PBMC stimulations than the N-terminal fragment ($p < 0.001$) (Fig. 1, *A* and *B*). rBet v 1 fragments as well as complete rBet v 1 induced dose-dependent release of IL-4, IL-5, IL-10, IL-13, and IFN-γ (Fig. 1*A*). Similar as in the proliferative responses, the C-terminal rBet v 1 fragment aa 75–160 induced significantly higher IFN-γ and IL-10 secretion compared with the N-terminal fragment ($p < 0.001$) (Fig. 1, *A* and *B*). The overall IL-4, IL-5, and IL-13 production was slightly higher for rBet v 1 fragment aa 1–74 ($p < 0.05$) compared with complete rBet v 1 or the C-terminal fragment (Fig. 1*B*). This divergence in cytokine profiles between the two fragments is clearly reflected by the significantly higher ratio of IL-4/IFN-γ or IL-13/IFN-γ secretion induced by rBet v 1 or rBet v 1 fragment aa 1–74 ($p < 0.05$ for IL-4/IFN-γ ratio; $p < 0.01$ for IL-13/IFN-γ ratio) (Fig. 2).

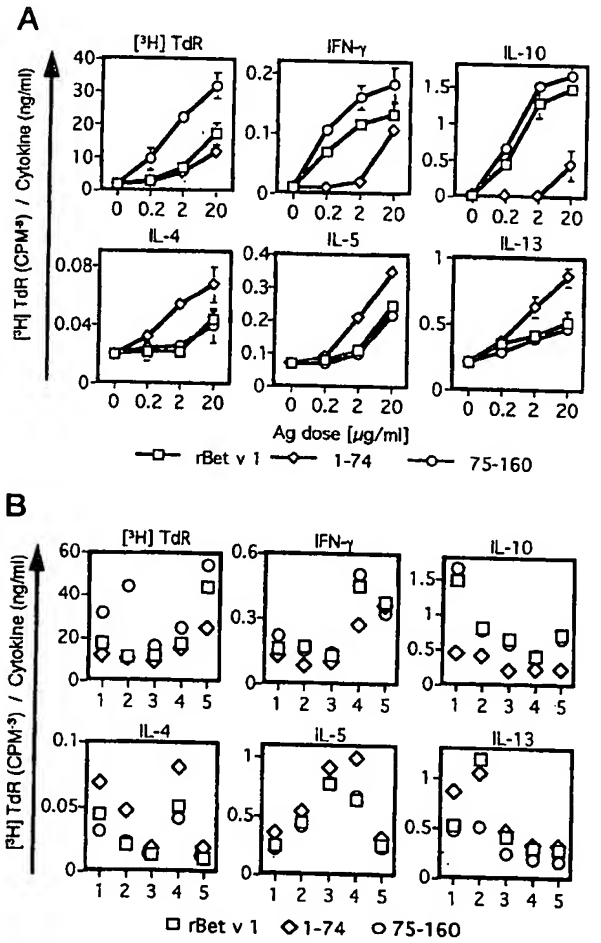

FIGURE 1. *A* and *B*, Induction of proliferation and cytokine production in PBMC from birch pollen-allergic patients with rBet v 1 and rBet v 1 fragments. PBMCs from five donors were stimulated with different doses of complete rBet v 1, rBet v 1 fragment aa 1–74, and rBet v 1 fragment aa 75–160. Supernatants were harvested 24 h later for IL-4 and 5 d later for IL-5, IL-10, IL-13, and IFN-γ. Thymidine incorporation was measured in parallel cultures at day 5. Results demonstrate means of two separate experiments in triplicate from a birch pollen-allergic patient (*A*). *B*, Thymidine incorporation and cytokine production of five patients with 20 μg/ml of rBet v 1 and the two fragments. Background thymidine incorporation in unstimulated cultures was <3153 cpm; IFN-γ was <0.023 ng/ml; IL-10 was <0.05 ng/ml; IL-4 was <0.021 ng/ml; IL-5 was <0.08 ng/ml; and IL-13 was <0.14 ng/ml.

Induction of Bet v 1-reactive IgG subclass responses in mice with rBet v 1 fragments

Groups of five mice each were immunized with rBet v 1 fragment aa 1–74 and rBet v 1 fragment aa 75–160, respectively. Table I displays the mean IgE and IgG subclass reactivities to rBet v 1 determined in serum samples obtained from each group before (0), after 12 wk (III) and 16 wk (IV) of immunization. We found that both rBet v 1 fragments induced IgE, IgG1, IgG2a, and IgG2b, but not IgG3 subclass responses to complete rBet v 1 (Table I). The IgE anti-rBet v 1 responses induced with the rBet v 1 fragments were rather low and could be detected only at serum dilutions of 1:10. IgG1 anti-rBet v 1 immunoreactivity was found at serum dilutions of 1:1000 and IgG2a/2b anti-rBet v 1 Abs could be detected at serum dilutions of 1:100 (Table I). In both serum samples obtained 12 and 16 wk after immunization the IgG1 anti-rBet v 1 Ab levels induced by the rBet v 1 fragment aa 1–74 were much lower (12 wk, 0.67; 16 wk, 1.36) than those induced by rBet v 1 fragment aa 75–160 (12 wk, 2.92; 16 wk, 2.88) (Table I). Likewise, we found that the IgG2a and IgG2b levels induced by the C-terminal fragment were higher than those induced by the N-terminal fragment (Table I). These findings, together with our observation that the anti-rBet v 1 aa 1–74 Ab levels induced by the first fragment were lower than the anti-rBet v 1 aa 75–160 Ab levels induced by the second fragment (data not shown) indicated a higher immunogenicity of rBet v 1 aa 75–160.

Hypoallergenic rBet v 1 fragments induce IgG Abs in rabbits and mice that cross-react with the Bet v 1 wild-type allergen and Bet v 1-homologous allergens

Rabbits and mice were immunized with rBet v 1 fragments, and serum samples were tested for cross-reactivity with rBet v 1 and Bet v 1-related plant allergens. Immunization of rabbits with purified rBet v 1 aa 1–74 or aa 75–160 gave rise to antisera that reacted with rBet v 1 wild type up to serum dilutions of 1:100,000. Rabbit antisera raised against rBet v 1 aa 1–74 (Fig. 3*A*, panel 1, *lane I*) and against rBet v 1 aa 75–160 (Fig. 3*A*, panel 2, *lane I*)

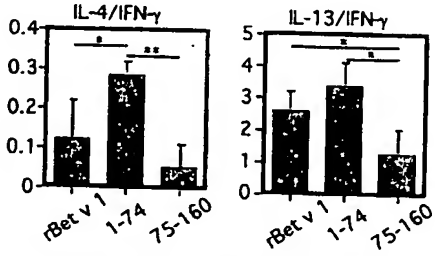

FIGURE 2. IL-4/IFN-γ and IL-13/IFN-γ ratios induced by rBet v 1 and rBet v 1 fragments. PBMC from five birch pollen-allergic patients were stimulated with 20 μg/ml of complete rBet v 1, rBet v 1 fragment aa 1–74, and rBet v 1 fragment aa 75–160. Supernatants were harvested 24 h later for IL-4 and 5 days later for IL-13 and IFN-γ. Results shown are mean ± SD of triplicate experiments from five birch pollen-allergic patients. *, $p < 0.05$; **, $p < 0.01$.

Table 1. *Mean IgE and IgG subclass responses to rBet v 1 were determined for groups of five mice each immunized with rBet v 1 aa 1–74 or rBet v 1 aa 75–160*[a]

	rBet v 1 aa 1–74			rBet v 1 aa 75–160		
	0	III	IV	0	III	IV
IgE (1:10)	0.09 ± 0.01	0.14 ± 0.05	0.22 ± 0.22	0.11 ± 0.01	0.26 ± 0.14	0.32 ± 0.29
IgG1 (1:1000)	0.06 ± 0.01	0.67 ± 1.04	1.36 ± 1.07	0.05 ± 0.01	2.92 ± 0.14	2.88 ± 0.16
IgG2a (1:100)	0.08 ± 0.004	1.23 ± 1.06	1.42 ± 0.75	0.08 ± 0.01	2.20 ± 1.06	1.55 ± 1.03
IgG2b (1:100)	0.05 ± 0.003	0.69 ± 0.84	0.87 ± 1.22	0.05 ± 0.004	1.27 ± 0.88	0.85 ± 0.44
IgG3 (1:100)	0.05 ± 0.003	0.11 ± 0.03	0.07 ± 0.02	0.06 ± 0.01	0.18 ± 0.10	0.09 ± 0.03

[a] The mean OD ± SD corresponding to the amount of bound Abs are displayed for IgE, IgG1, IgG2a, IgG2b, and IgG3 for serum samples obtained in each group shortly before (0), 12 wk (III), and 16 wk (IV) after the first immunization.

reacted with nitrocellulose-blotted rBet v 1, natural Bet v 1, and Bet v 1-related allergens present in pollens of alder, hazel, hornbeam, and oak, as well as in apples (Fig. 3*A*). The rabbits' preimmune sera showed no reactivity to the nitrocellulose-blotted allergens (Fig. 3*A*, *lane P*). The intensity of reactivity to Bet v 1-homologous allergens was in parallel to the degree of sequence homology. For example, the major allergen from alder pollen, Aln g 1, (41), which has a higher degree of sequence identity to Bet v 1 (81.1%) than the major hazel pollen allergen Cor a 1 (42) (72.3%), reacted stronger with the rabbit anti-rBet v 1 fragment Abs than Cor a 1 (Fig. 3*A*). Sera from all immunized mice contained IgG Abs to complete rBet v 1 wild type, however, with a somewhat lower titer (1:1000) than the rabbit antisera. As exemplified in Fig. 3*B*, mouse anti-rBet v 1 aa 1–74 (panel 1, *lane I*) and mouse anti-rBet v 1 aa 75–160 (panel 2, *lane I*) reacted with nitrocellulose-blotted rBet v 1, natural Bet v 1, and Bet v 1-related allergens, exhibiting a high degree of sequence identity with Bet v 1 (e.g., alder, Aln g 1 81.1%; hornbeam, Car b 1 73%) (Fig. 3*B*). No (apple, Mal d 1, 56.3%) or weak responses (hazel, Cor a 1, 72.3%, oak) to allergens with low sequence identity to Bet v 1 were found (data not shown). No reactivity was observed when blotted allergens were incubated with the corresponding mouse preimmune sera (Fig. 3*B*, *lane P*).

rBet v 1 fragment-induced mouse and rabbit Abs inhibit IgE binding of allergic patients to complete rBet v 1 wild type

Next we investigated whether rBet v 1 fragment-induced IgG Abs can inhibit the IgE binding of patients to the complete Bet v 1 wild-type allergen. Preincubation of ELISA plate-coupled rBet v 1 with a mouse serum raised against rBet v 1 fragment aa 1–74 inhibited the IgE binding of allergic patients (Table II, patients 1–4) to Bet v 1 between 45 and 83% (59.5% mean inhibition) (Table II). A mouse serum raised against rBet v 1 fragment aa 75–160 inhibited IgE binding to rBet v 1 of the same patients between 73 and 78% (74.8% mean inhibition).

In another series of experiments we analyzed rabbit anti-rBet v 1 fragment aa 1–74 and anti-rBet v 1 fragment aa 75–160 for their ability to block IgE binding of a representative number (n = 70) of birch pollen-allergic patients' sera to Bet v 1. In one series of experiments performed with 35 sera from Bet v 1-allergic patients, we found that rabbit anti-rBet v 1 aa 1–74 and anti-rBet v 1 aa 75–160 Abs inhibited human IgE binding to rBet v 1 between 29 and 89% (62.1% mean inhibition) and between 22 and 76% (56.8% mean inhibition), respectively (data not shown). The mean inhibition obtained with a mixture of both antisera was slightly higher (64.1% mean inhibition) than that found for each antiserum (data not shown). Next, we compared the inhibitory capacity of the anti-fragment antisera with that of an antiserum raised against complete rBet v 1 in 35 additional Bet v 1-allergic patients (Table III). Rabbit anti-rBet v 1 aa 1–74 and anti-rBet v 1 aa 75–160 Abs inhibited human IgE binding to rBet v 1 between 33 and 88% (67% mean inhibition) and between 10 and 81% (54% mean inhibition), respectively (Table III). Rabbit anti-rBet v 1 Abs blocked human IgE binding to rBet v 1 between 17 and 86% (52% mean inhibition) (Table III). We also noted that the degree of inhibition was not associated with the levels of Bet v 1-specific IgE Abs present in the sera from allergic patients (data not shown).

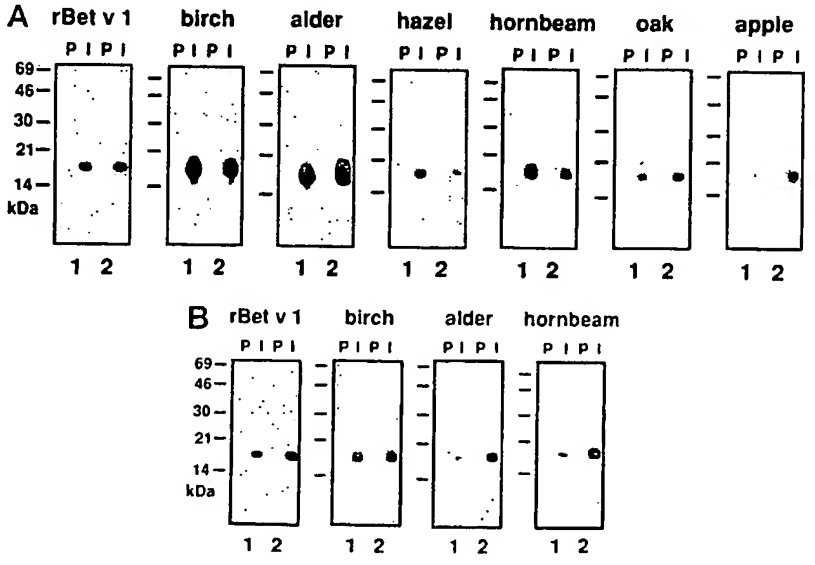

FIGURE 3. *A* and *B*, Cross-reactivity of mouse and rabbit anti-rBet v 1 fragment Abs with Bet v 1 and Bet v 1-homologous allergens. Nitrocellulose-blotted rBet v 1 and natural allergen extracts were exposed to rabbit sera (*A*; rBet v 1, birch, alder, hazel, hornbeam, oak, apple) or mouse sera (*B*; rBet v 1, birch, alder, hornbeam) raised against purified rBet v 1 fragments (*panel 1*, aa 1–74; *panel 2*, aa 75–160) (*lane I*) or to the corresponding preimmune sera (*lane P*).

Table II. *Mouse anti-rBet v 1 fragment antisera inhibit allergic patient's IgE binding to Bet v 1*[a]

Patient	% Inhibition of IgE Binding			
	1	2	3	4
Mouse anti-rBet v 1 aa 1–74	51	60	45	83
Mouse anti-rBet v 1 aa 75-160	74	78	74	73

[a] The percentage inhibition of serum IgE binding of four patients to ELISA plate-bound Bet v 1 after preincubation with a mouse serum raised against rBet v 1 fragment aa 1–74 and against rBet v 1 fragment aa 75–160 is displayed.

rBet v 1 fragment-induced rabbit Abs inhibit IgE binding of allergic patients to natural Bet v 1 and Bet v 1-related Fagales *pollen allergens*

Natural pollen extracts represent allergen mixtures that also contain isoallergenic variants of certain allergens (43). Therefore, we were interested to investigate whether rabbit anti-rBet v 1 fragment antisera can also inhibit IgE binding of allergic patients to birch pollen extract and to pollen extracts from botanically related trees (alder, hornbeam, and oak) containing Bet v 1-homologous allergens. As exemplified in Table IV, we found that rabbit anti-rBet v 1 fragment antisera strongly inhibited IgE binding to birch pollen extract (Table IV, rabbit anti-rBet v 1 fragment aa 1–74, 64 and 54%; rabbit anti-rBet v 1 fragment aa 75–160, 58 and 49%). An albeit lower but substantial inhibition of IgE binding, ranging between 10 and 56%, to pollen extracts from alder, hornbeam, and oak, was observed (Table IV).

Discussion

More than 95% of birch pollen-allergic patients are sensitized against the major birch pollen allergen, Bet v 1 (17, 18). Recently we produced by genetic engineering two recombinant fragments of Bet v 1 comprising aa 1–74 and aa 75–160 (26). The recombinant Bet v 1 fragments had lost their IgE binding capacity due to lack of their conformation and thus exhibited a >100-fold reduced allergenic activity when tested for their ability to elicit immediate skin reactions in patients (26, 28, 29). Although T cell epitopes of several Bet v 1-specific T cell clones seemed to be present on the rBet v 1 fragments (26), no information was available as to whether the derivatives would be capable of inducing significant lymphoproliferative responses in birch pollen-allergic patients. In this study, we show that the recombinant Bet v 1 fragments contain the relevant Bet v 1-specific T cell epitopes by comparing their ability with that of complete rBet v 1 to induce proliferation of PBMC from birch pollen-allergic patients. The strong lymphoproliferative responses induced by the rBet v 1 fragments can be explained by the fact that both fragments together resemble the T cell epitope repertoire of complete rBet v 1 and the fact that they were engineered by selecting the breakpoint between the fragments outside known Bet v 1-specific T cell epitopes (26, 44). Our finding that rBet v 1 fragments were potent inducers of T cell responses are in line with a previous study demonstrating that changes in antigenic conformation to a non-IgE binding structure increases the T cell stimulation capacity and induces higher proliferation and IFN-γ production leading to decreased IL-4/IFN-γ and IgE/IgG ratios (45).

We consider the presence of relevant T cell epitopes on the rBet v 1 fragments as important for their potential use for immunotherapy of birch pollen allergy as there is evidence that induction of Th1 responses or T cell tolerance is associated with a successful outcome of immunotherapy (34, 35, 46–49). IL-10-induced peripheral T cell anergy and reactivation by microenvironmental cytokines were shown to be critical steps in specific immunotherapy (35, 49). In this context, induction of higher IL-10 levels by the second rBet v 1 fragment suggests that this particular fragment may also exert tolerogenic activity. Interestingly, the same fragment induced stronger IFN-γ secretion in PBMC of all patients tested and, in a murine model of nasal tolerance induction, was found to suppress Bet v 1-specific IgE production and airway hyperresponsiveness (50).

Although each of the recombinant Bet v 1 fragments had lost IgE binding capacity due to lack of structural fold, they were able to induce Bet v 1-specific Abs in mice and rabbits. The fact that the fragments failed to induce high IgE levels could be as much a feature of the adjuvant used as of the Ag. However, we consider it important that the fragment-induced Abs recognized the complete, folded wild-type allergen as well as Bet v 1-homologous allergens from other plants and inhibited IgE binding of allergic patients to the wild-type allergens. This finding indicates that rBet v 1 fragments contain sufficient sequence motifs to induce blocking Ab responses against the complete wild-type allergen in vivo. Because both rBet v 1 fragments represent unfolded molecules (26), it is unlikely that they induced IgG Abs to conformational (i.e., discontinuous) Bet v 1 IgE-defined epitopes. Therefore, their ability to block human IgE recognizing conformational Bet v 1 epitopes may be explained in two ways. One possibility is that the fragment-induced Abs bound in close proximity to the IgE-defined epitopes and thus exhibited steric hindrance of human IgE binding. A second, not mutually exclusive, possibility is that the fragment-induced Abs recognized continuous portions within discontinuous IgE epitopes and thus were able to prevent IgE binding.

Recently, increasing evidence has accumulated that the induction of blocking Abs of the IgG isotype may be important for the successful outcome of immunotherapy in patients. It has been shown that allergen-specific IgG Abs can inhibit the IgE-mediated release of biological mediators from effector cells and thus may prevent immediate symptoms (37, 51–53). Although not yet proven, it is also possible that blocking Abs may capture allergens during natural allergen exposure and thus prevent allergen-induced

Table III. *Rabbit anti-rBet v 1 fragment and anti-rBet v 1 antisera inhibit allergic patient's binding to rBet v 1 wild type*[a]

Patient	% Inhibition of IgE Binding																																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Rabbit anti-rBet v 1 aa 1–74	78	76	71	45	63	70	52	75	81	84	47	73	72	61	40	64	39	66	40	80	85	87	69	88	83	64	69	75	33	86	75	80	66	67	38
Rabbit anti-rBet v 1 aa 75–160	45	62	45	10	32	55	47	66	57	62	43	57	62	22	49	42	21	53	33	69	71	80	58	71	76	66	54	74	28	81	66	73	54	65	38
Rabbit anti-rBet v 1	62	83	71	41	76	63	70	58	57	75	58	41	44	17	54	69	26	44	23	67	79	86	46	80	53	23	21	32	26	51	47	43	49	43	29

[a] The percentage inhibition of IgE binding after preincubation of rBet v 1 with rabbit anti-rBet v 1 aa 1–74, rabbit anti-rBet v 1 aa 75–160, and with a rabbit anti-rBet v 1 antiserum is displayed for sera from 35 birch pollen-allergic patients (1–35).

Table IV. *Rabbit anti-rBet v 1 fragment antisera inhibit patient's IgE binding to natural tree pollen extracts*[a]

Patient	% Inhibition of IgE Binding							
	Birch		Alder		Hornbeam		Oak	
	A	B	A	B	A	B	A	B
Rabbit anti-rBet v 1 aa 1–74	64	54	10	12	27	30	20	11
Rabbit anti-rBet v 1 aa 75–160	58	49	56	40	16	12	35	15

[a] ELISA plate-coated pollen extracts from birch, alder, hornbeam, and oak were preincubated with rabbit anti-rBet v 1 aa 1–74 and aa 75–160. The percentage of inhibition of IgE binding is displayed for two allergic patients (A, B).

rises of IgE production in patients. Experimental support for the beneficial role of blocking Abs regarding late allergic symptoms comes from two other recent observations that 1) sera containing therapy-induced allergen-specific IgG Abs were found to suppress IgE-mediated presentation of allergens to T cells and thus to reduce T cell proliferation and cytokine release (54); and 2) hypoallergenic rBet v 1 derivatives lacking the capacity to induce cross-linking of effector cell-bound IgE Abs also failed to activate eosinophils, suggesting that allergen-induced IgE-mediated activation of eosinophils is operative in vivo and might be suppressed by blocking Abs (30).

Our observation that hypoallergenic rBet v 1 fragments induced strong lymphoproliferative responses and, despite lacking IgE epitopes, could induce blocking Ab responses in vivo suggests that both molecules might represent candidate molecules for immunotherapy of birch pollen allergy. Using recombinant birch pollen allergens for component-resolved diagnosis (15), patients who are not sensitized to Bet v 1 or who are sensitized to birch pollen allergens other than Bet v 1 can be excluded from treatment. Different regimens of immunotherapy may be considered for the rBet v 1 fragments (e.g., rush immunotherapy, sublingual immunotherapy, and tolerance induction) but we suggest to administer the molecules bound to adjuvants by conventional injection immunotherapy because it was shown that administration of unbound peptides, even when they contain exclusively T cell epitopes and lack allergenic activity, can elicit systemic side effects (i.e., asthma) (55).

We anticipate several advantages from an immunotherapy protocol with hypoallergenic rBet v 1 fragments. Selection of patients by component-resolved diagnosis will allow component-resolved immunotherapy with the disease-eliciting allergen and thus prevent de novo sensitization against other components present in natural allergen-extracts (15, 53). As rBet v 1 fragments exhibited a >100-fold reduced allergenic activity, we expect fewer side effects and think that higher doses can be administered. The latter will likely favor the rise of a Bet v 1-specific Th1 immune response, which is accompanied by the induction of high levels of Bet v 1-specific blocking Abs belonging to the IgG isotype. The fact that the rBet v 1 fragment-induced Abs cross-reacted with Bet v 1-homologous allergens from other plants even gives rise to the hope that also birch pollen-related allergies may be treated with the new vaccine. In this context it has been shown that conventional immunotherapy with birch pollen extract was effective for the treatment of allergies to other *Fagales* pollens (e.g., alder, hazel) (56) and birch pollen-related plant food allergies (e.g., apple) (57). Clinical trials with the rBet v 1 fragment-based vaccine will now have to follow. Such trials will allow the study of mechanisms underlying injection immunotherapy at a component level and will determine whether a rBet v 1 fragment-based vaccine is clinically effective for the treatment of birch pollen allergy.

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(57) Abstract

An immunogen derived from a protein allergen comprises: a) a polymeric form of a non-anaphylactic immunogenic recombinant fragment of the protein allergen, said fragment containing an IgG epitope partly but not wholly overlapping an IgE epitope of the protein allergen, in which polymeric form said fragment constitutes the monomeric units; or b) a recombinant polymeric form of said protein allergen in which the protein allergen constitutes the monomeric units. The immunogen may be used for in vitro diagnosis of type I allergy and hyposensitization.

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NON-ANAPHYLACTIC FORMS OF ALLERGENS AND THEIR USE

Technical field and background

The present invention concerns non-anaphylactic forms of protein allergens and the use of the forms for hyposensitization and for determining antibodies (IgA, IgD, IgE, IgG, IgM) directed against the allergen, for instance in the context of diagnosing in vitro type I allergy (IgE mediated allergy). The invention also concerns a method for hyposensitization of a mammalian individual, typically a human individual, suffering from type I allergy against a protein allergen.

The invention primarily concerns treating and diagnosing humans.

By a protein allergen is meant any protein/polypeptide causing a type I mediated allergic reaction. Thus the term encompasses any naturally occurring protein allergen including the smallest fragments thereof that will cause a type I allergic reaction in a mammal, most importantly humans.

In April 1997, the present inventors published an article dealing with non-anaphylactic fragments of the Bet v 1 allergen. See Vrtala et al., "Conversion of the major birch pollen allergen, Bet v 1, into two non-anaphylactic T cell epitope containing fragments", J. Clin. Invest. 99(7) April 1997, 1673-1681.

Type I allergy represents a major health problem in industrialised countries where more than 20 % of the population suffer from Type I allergic reactions (allergic rhinitis, conjunctivitis, allergic asthma and anaphylactic shock) (Kaplan (ed) Allergy. Churchill Livingstone, New York (1985)). Environmental proteins from pollen, mites and animal dander belong to the major components which induce release of biological mediators (e.g. histamine) by

crosslinking effector cell (mast cell, basophil) bound specific IgE antibodies. The production of specific IgE from B-cells is stimulated by allergen specific T-helper cells which in their majority belong to the TH2 type (Romagnani, Immunol. Today 13 (1992) 379-381). Therapy of Type I allergic diseases is currently performed by pharmacological treatment and by specific immunotherapy. Specific immunotherapy has been established already early in this century (Noon, Lancet 1 (1911) 1572-1573) and involves the systemic application of increasing doses of allergens for extended periods. Although specific immunotherapy is recognized as effective treatment, the occurrence of anaphylactic side effects represents one of the major disadvantages of this therapy. To reduce anaphylactic reactions the use of T-cell epitopes has recently been proposed for allergen specific immunotherapy (Briner et al., Proc. Natl. Acad. Sci. USA 90 (1993) 7608-7612, and Norman, Curr. Opin. Immunol. 5 (1993) 986-973).

Allergens harbour a great variety of different T-cell epitopes (Ebner et al., J. Immunol 150 (1993) 1047-1054; Joost-van-Neerven et al., J. Immunol. 151 (1993) 2326-2335; and Schenket al., J. Allergy Clin. Immunol. 96 (1995) 986-996) which may overlap with continuous IgE-epitopes. To prevent crosslinking of effector cell (mast cell, basophil) bound IgE and mediator release, T-cell epitopes and IgE epitopes need to be dissected. Following the concept of converting a major allergen into a T-cell vaccine, the present inventors selected Bet v 1 (Breiteneder et al., EMBO J. 8 (1989) 1935-1938), the major birch pollen allergen, as a model.

Bet v 1 was selected because epitope analysis indicated that it forms conformational IgE epitopes (Visco et al., J. Immunol. 157 (1996) 956-962; and Laffer et al., J. Immunol. 157 (1996) 4953-4962). In addition, Bet v 1 represents one of the most common allergens which is recognized by 95% of

tree pollen and food allergic individuals and almost 60% of them are sensitized exclusively against Bet v 1 (Jarolim et al., Allergy 44 (1989) 385-394). The cDNA coding for Bet v 1 has recently been isolated (Breiteneder et al., EMBO J. 8 (1989) 1935-1938) and recombinant Bet v 1 was expressed in Escherichia coli (Valenta et al., J. Allergy Clin. Immunol. 88 (1991) 889-894; and Ferreira et al., J. Biol. Chem. 268 (1993) 19574-19580). Recombinant Bet v 1 has been shown to possess an IgE-binding capacity similar to that of natural Bet v 1 and shares IgE as well as T-cell epitopes with Bet v 1 homologous proteins present in pollen from various trees and plant derived foods (Ebner et al., J. Allergy Clin. Immunol. 95 (1995) 962-969; Ebner et al., J. Immunol. 150 (1993) 1047-1054; and Schenk et al., Eur. J. Biochem. 224 (1994) 717-724). The biological activity of the recombinant Bet v 1 has been demonstrated by histamine release experiments and by skin prick testing of allergic patients (Valenta et al., J. Allergy Clin. Immunol. 91 (1993) 88-97; Pauli et al., J. Allergy Clin. Immunol. 98 (1996) 1100-1109; and Menz et al., Clin. Exp. Allergy 26 (1995) 50-60).

The invention.

A first aspect of the present invention is an immunogen derived from a protein allergen. It has a strongly reduced anaphylactic ability compared to the protein allergen from which it derives and will therefore in the context of the present invention be called non-anaphylactic. The immunogen is characterized in that it comprises:

a) a polymeric form of a non-anaphylactic immunogenic recombinant fragment of the protein allergen, said fragment containing an IgG epitope partly but not wholly overlapping an IgE epitope of the protein allergen, in which polymeric form the fragment constitutes the monomeric units; or

b) a recombinant polymeric form of said protein allergen in which the protein allergen constitutes the monomeric units.

Thus, in accordance with the present invention, the IgE-binding capacity of an allergen is reduced by genetic fragmentation or polymerization.

In the above-mentioned non-anaphylactic (or hypoallergic) allergen fragment, the IgE epitope epitope has been broken up by fragment formation. By the term "a broken up IgE epitope" is meant that the fragment formation has resulted in a fragment that only contains a part of the corresponding IgE epitope present in the starting protein allergen. The epitopes in question may be either conformational or linear, with particular emphasis for the IgE epitope being conformational in case of a fragment according to polymeric form (a) above. Compare Bet v 1 fragments aa 1-74 and 75-160 as described in the experimental part herein and by Vratala et al., J. Clin. Invest. 99(7) April 1997, 1673-1681.

Preferably, the fragment is larger than 15 amino acids. The maximum size of the fragment varies depending on the specific allergen. Usually, the maximum size may be said to be the largest fragment of the allergen that can be produced without yielding anaphylactic reactions corresponding to 10% of the reaction induced by the complete allergen (as determined by skin testing or basophil histamine release).

By polymeric forms means that the immunogen typically comprises 2-10 of the monomeric units defined in (a) and (b) above. At the priority date results had been obtained with polymeric forms containing 2, 3 and 4 monomeric units.

The immunogens (a) and (b) may be produced by recombinant techniques to directly give the polymeric forms according to (a) and (b). For (a) the polymeric form may also be

accomplished by covalently linking two or more identical recombinant fragment molecules, optionally to a common carrier molecule. In the final immunogen that is to be used for hyposensitization therapy or in vitro assays, the polymeric forms according to (a) and (b) may have been linked to a carrier in order to increase the immunogenicity. In case this carrier is a protein and one wants to have a linear immunogen it is possible to produce the immunogen in one step by expression of the corresponding gene construct in the appropriate host cell, such as a procaryotic (e.g. E. coli) or eucaryotic (yeast or a mammalian cell line) cell. See further Scheiner O and Kraft D, Allergy 50 (1995) 384-391; and Valenta R and Kraft D, Current Opinion in Immunology 7 (1995) 751-756.

By the use of recombinant techniques it is easy to introduce oligopeptide linkers between each monomeric unit of the polymeric forms of the immunogen according to (a) and (b), respectively. Suitable amino acid residues in the linker may be selected among hydrophobic or hydrophilic or among basic, acid or neutral amino acids. Hydrophobic amino acids are Trp, Gly, Ala, Phe, Pro, Met, Val, Leu, and Ile. Hydrophilic amino acids are for instance Gln, Ser, Gly, Glu, Pro, His and Arg. The length of the oligopeptide linker typically is an integer in the interval 0-30, such as in the interval 0-10, amino acid residues. At the priority date, the preferred linker was the tripeptide Leu-Val-Pro.

In the experimental part the invention is illustrated with the birch pollen allergen Bet v 1.

A second aspect of the invention is a specific hyposensitization therapy. This therapy may be performed as known in the art for protein allergens and encompasses administering repeatedly to the mammal, typically a human individual, suffering from type I allergy against the protein allergen an immunogen that is capable of raising an

IgG immune response against the protein allergen. Administration may be done systemically, for instance by injection, infusion, etc, but also the oral route has been suggested in order to expose the intestinal part of the immune system. The immunogen may be admixed with suitable adjuvants such as aluminium oxide. Se further Norman PS, "Current status of immunotherapy for allergies and anaphylactic reactions" Adv. Internal. Medicine 41 (1996) 681-713.

A third aspect of the invention is to use the immunogen of the first aspect, in particular according to form (b), as an antigen in an immunoassay for detecting specific antibodies of the IgA, IgD, IgE, IgG or IgM class directed against the protein allergen or protein allergens from which the immunogen derives. Appropriate assay variants involve formation of a ternary immune complex between the immunogen, sample antibody and an antibody directed against the Ig-class of interest. The sample may be any Ig-containing biological fluids, for instance a blood derived sample (serum, plasma, whole blood), CSF, etc.

The invention will be defined in the attached claims that are part of the specification. The invention will now be illustrated by three non-limiting Examples.

EXPERIMENTAL PART

Example 1. Bet v 1 polymers

Construction of the Bet v 1-polymers

The Bet v 1-cDNA (Breiteneder et al., "The gene coding for the major birch pollen allergen Bet v 1 is highly homologous to a pea resistance response gene", EMBO J. 8 (1989) 1935-1938) was PCR-amplified with the following oligonucleotide primers:

Bet v 1-dimer:

For construction of the first Bet v 1-segment:

Sequence Id No 1:

5'GAG GAA TTC *CAT ATG* GGT GTT TTC AAT TAC3'

Eco RI Nde I

Sequence Id No 2:

5'CGG GGT ACC AAG TTG TAG GCA TCG GAG TG3'

Kpn I

For construction of the second Bet v 1-segment:

Sequence Id No 3:

5'CGG GGT ACC GAT GGG TGT TTT CAA TTA C3'

Kpn I

Sequence Id No 4:

5'CCG GAA TTC CCG *CTC GAG* CTA TTA GTT GTA GGC ATC GGA GTG3'

Eco RI Xho I

Bet v 1-trimer:

First Bet v 1-segment:

The same primers were used as for construction of the first segment of Bet v 1-dimer.

Second Bet v 1-segment:

Sequence Id No 5:

5'CGG GGT ACC GAT GGG TGT TTT CAA TTA C3'

Kpn I

Sequence Id No 6:

5'CGG AAT TCA *CTA GTG* GGT TGT AGG CAT CGG AGT G3'

Eco RI Spe I

Third Bet v 1-segment:

Sequence Id No 7:

5'CCG GAA TTC GGA *CTA GTA* ATG GGT GTT TTC AAT TAC3'

Eco RI Spe I

Sequence Id No 8:

5'CGG AAT TCG TTG TAG GCA TCG GAG TG3'

Eco RI

Protocol for PCR-amplification. Reaction mix (GeneAmp PCR kit, Perkin Elmer, Branchburg, N.J. USA): 44µl H_2O_{dd}, 10xl 10x PCR buffer, 4µl 5mM dATP, 4µl 5mM dCTP, 4µl 5mM dGTP, 4µl 5mM dGTP, 4µl 25mM $MgCl_2$, 3µl 10xM primer 1, 3µl 10xM primer 2, 10µl 1ng/µl Bet v 1. 10x PCR-buffer: 100mM Tris-HCl, pH 8.3, and 500 mM KCl. The reaction mixture was heated for 5 minutes at 94°C, afterwards 35 cycles of 1min at 94°C, 2min at 40°C, and 3 min at 72°C were performed. During the first cycle 10µl of AmpliTaq DNA Polymerase (2.5 U/10µl) were added.

After PCR-amplification, the PCR-products were digested with the corresponding restriction enzymes. Primers which contained additional Eco RI sites, were digested first with Eco RI to facilitate subcloning. Digested fragments were purified using Nick columns (Pharmacia Biotech AB, Uppsala, Sweden), and ligated into pET-17b plasmids (Novagen, Madison, USA). The plasmid, containing the first Bet v 1-segment, was further digested with Kpn I/Xho I in the case of Bet v 1-dimer, or with Kpn I/Spe I in the case of Bet v 1-trimer, to obtain vectors, in which the second Bet v 1-segments could be incorporated. In the case of Bet v 1-trimer, this construct was further digested with Spe I/Eco R I and the third Bet v 1-segment was added.

Expression and purification of recombinant Bet v 1-polymers
Recombinant Bet v 1-dimer and recombinant Bet v 1-trimer were expressed in E. coli BL21 (DE3) by induction with 0.5 mM isopropyl beta-thiogalactopyranoside at an OD600 of 0.5-0.8 in liquid culture (LB-medium) for 5h at 37°C. E. coli cells were the harvested by centrifugation and washed to remove the culture medium.

LB-medium: 10g sodium chloride, 10g peptone, 5g yeast extract, pH 7.5 with NaOH, autoclaved prior to use.

Purification. Recombinant Bet v 1-polymers were expressed as inclusion bodies and isolated as described (Vrtala et al., "Immunologic characterization of purified recombinant timothy grass pollen (Phleum pratense) allergens (Phl p 1, Phl p 2, Phl p 5)", J. Allergy Clin. Immunol. 97 (1996) 781-786. Inclusion bodies were solubilized with 8M urea, 10mM Tris, pH 8, 1mM EDTA, 5mM beta-mercaptoethanol, diluted with 10mM Tris, pH 8, to a concentration of 6M urea and centrifuged for 15min at 10,000g to remove insoluble material. The supernatant, containing the recombinant protein, was dialyzed to a final concentration of 2M urea. After centrifugation (15min, 10,000g), the supernatant was applied to a column packed with DEAE Sepharose (Pharmacia Biotech AB, Uppsala, Sweden), and the protein was eluted with a 0-0.5M NaCl-gradient. Fractions, containing the recombinant protein which was > 80% pure, were dialyzed against 6M urea, 10mM NaH_2PO_4, pH 4.8, and rechromatographed on a column packed with SP Sepharose (Pharmacia Biotech AB, Uppsala, Sweden). Fractions, containing recombinant Bet v 1-dimer or recombinant Bet v 1-trimer of > 95% purity were dialyzed against 10mM Tris, pH 7.5 and stored at -20°C until used.

Results of studies on Bet v 1 polymers

Construction of the Bet v 1 polymers. It is referred to the scheme with sequences and vector figures at the end of the descriptive part. The Bet v 1-cDNA (Breiteneder et al., EMBO J. 8 (1989) 1935-1938) was PCR-amplified with oligonucleotide primers containing different restriction enzyme cleavage sites. The PCR-products were then ligated as indicated in the scheme and subcloned into the plasmid pET-17b (Novagen, Madison, USA).

Figure 1. Coomassie stained SDS-PAGE gel showing purified recombinant Bet v 1-monomer and Bet v 1-polymers

Lane M: Molecular weight marker; lane 1 contains 3µg purified, recombinant Bet v 1 monomer, lane 2 3µg purified, recombinant Bet v 1-dimer, and lane 3 3µg purified recombinant Bet v 1-trimer.

Result: The purified proteins were more than 95% pure. The dissolved proteins were separated from insoluble material by high speed centrifugation prior to loading the samples.

Figure 2. IgE-reactivity of birch-pollen allergic patients with nitro-cellulose-blotted purified recombinant Bet v 1-monomer, dimer and trimer.

Purified recombinant Bet v 1-monomer, dimer and trimer were separated by SDS-PAGE and blotted onto nitro-cellulose. Sera from 8 different birch pollen allergic patients (lanes 1-8) and serum from a non-allergic person (lane 9) were used to detect the blotted allergens. Bound IgE was detected with ^{125}I labelled anti-human >IgE antibodies (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) and visualised by autoradiography.

Result: The IgE-binding capacity of nitrocellulose-blotted Bet v 1-polymers was comparable to that of Bet v 1-monomer.

Figure 3: Determination of IgE-reactivity of sera from birch pollen allergic patients with Bet v 1-monomer and polymers by ELISA.

Sera from 4 birch-pollen allergic patients (A-D) were diluted 1:2 (1), 1:10 (2), 1:20 (3), 1:40 (4) and 1:80 (5) and tested for IgE-reactivity with purified, recombinant Bet v 1-monomer, Bet v 1-dimer and Bet v 1-trimer. The OD-values are displayed on the y-axis.

Result: Serum IgE from allergic patients bound to Bet v 1-polymers in a comparable manner as to Bet v 1-monomer.

Figur 4. Inhibition of IgE-binding to recombinant Bet v 1-monomer using Bet v 1-polymers.

Sera from 4 birch-pollen allergic patients (A-D) were preincubated with different concentrations (5µg, 500ng, 50ng and 5ng) of purified, recombinant Bet v 1-monomer, Bet v 1-dimer and Bet v 1-trimer. The preincubated sera were then tested for IgE-reactivity to purified, recombinant Bet v 1-monomer by ELISA. The optical densities (OD) are displayed on the y-axis.

Result: IgE-binding to Bet v 1-monomer is inhibited by increasing concentrations of the Bet v 1-polymers in a dose dependent manner. The amounts of Bet v 1-polymers needed for inhibition at certain concentrations (50 ng versus 5 ng) was however approximately tenfold higher compared to the monomer.

Figure 5. Serum IgG_1-reactivity of Bet v 1-polymer immunized mice with recombinant Bet v 1.

8 Balb/c mice were immunized monthly with 5µg purified, recombinant Bet v 1-dimer and $Al(OH)_3$ as adjuvant, 8 Balb/c mice were immunized monthly with 5µg purified, recombinant Bet v 1-trimer-$Al(OH)_3$ and blood samples were taken after each immunization. Serum samples obtained after weeks 19 and 25 of immunization and serum taken before immunization (preimmune serum 0 =) were diluted 1:1000 and tested for IgG_1-reactivity with purified, recombinant Bet v 1-monomer in an ELISA. The symbols represent the OD-values that correspond to the IgG_1-binding of the 8 different Bet v 1-dimer or Bet v 1-trimer mice.

Result: The Bet v 1-polymers are able to induce high levels of IgG_1-antibodies, which crossreact with Bet v 1-monomer.

Figure 6. Capacity of recombinant Bet v 1-polymers to induce histamine release.

Granulocytes from a birch pollen allergic patient were incubated with increasing concentrations (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml) of purified, recombinant Bet v

1-monomer, Bet v 1-dimer, Bet v 1-trimer, Bet v 1-tetramer and anti-IgE antibodies as positive control. Histamine release in the cell free supernatant was measured by RIA (Immunotech, Marseille) and is expressed as percentage of total histamine release.

Result: Bet v 1-dimer induced a slightly reduced histamine release from patients' basophils compared to the monomer, whereas Bet v 1-trimer and tetramer had an approximately 100 fold reduced capacity to induce histamine release. In the donors tested, Bet v 1-monomer induced maximal histamine release at a concentration of 0.01 µg/ml, Bet v 1-trimer and tetramer at a concentration of 1 µg/ml.

Table 1. Proliferation of Bet v 1 specific T-cell clones with recombinant Bet v 1-polymers.

The full table is given at the end of the descriptive part. T-cell clones from different pollen allergic donors (column 2 shows the initials of the donors) with specificity for different Bet v 1 epitopes (in column 1 the position of the epitopes are indicated) were incubated with purified, recombinant Bet v 1-monomer (column 4), Bet v 1-dimer (column 5), Bet v 1-trimer (column 6) and Bet v 1-tetramer (column 7). As negative control, clones were tested with medium alone (column 3). Proliferation was determined by ^{3}H Thymidine uptake and is displayed as counts per minute (cpm) (columns 3-7).

Result: Bet v 1-polymers and Bet v 1-monomer induced comparable proliferation of specific T cell clones.

Table 2. Skin testing with recombinant Bet v 1-monomer and polymers.

The full table is given at the end of the descriptive part. 6 birch-pollen allergic individuals and 4 non-allergic control individuals were skin prick tested on their forearms with natural birch pollen extract, histamine as positive control and with 10µg/ml and 100µg/ml of purified, recombinant Bet v 1-monomer, Bet v 1-dimer and Bet v 1-

trimer. The mean wheal diameters (DM) are displayed in the table.

Result: Bet v 1-dimer induced an approximately 10-fold reduced skin reaction in allergic patients compared to Bet v 1-monomer, whereas Bet v 1-trimer induced in some patients no wheal reactions at all, up to a concentration of 100µg/ml. The wheal reaction increased dose dependently with the protein concentrations. The non-allergic control individuals displayed only skin reactions with histamine but not with the Bet v 1-preparations. Both the histamine release assays and the skin tests indicate that the Bet v 1-polymers have a greatly (up to 100 fold) reduced anaphylactic activity compared to Bet v 1-monomer. The reduction of anaphylactic potential is proportional to the degree of polymerization.

Summary - studies on Bet v 1 polymers.

We expressed in pET 17b plasmids (Novagen, Madison, USA) Bet v 1 as dimer, trimer and tetramer. The Bet v 1-polymers were expressed at high levels in E. coli BL21 (DE3) (Novagen, Madison, USA) and purified to homogeneity. The Bet v 1-polymers retained their IgE-binding capacity, as was shown by immunoblotting and by ELISA. T-cell clones from birch allergic donors, with specificity for Bet v 1 proliferated upon incubation with all the polymers, indicating that the polymers contain the relevant T-cell epitopes of Bet v 1. Bet v 1-trimer and tetramer had an approximately 100 fold reduced capacity to induce histamine release from patients' basophils and a greatly reduced anaphylactic potential as evaluated by skin testing. Because of the reduction of their anaphylactic activity the Bet v 1-polymers may be considered as safe tools for specific immunotherapy of tree pollen and associated food allergy. Allergic patients may be treated with high doses of these derivatives with reduced risk of anaphylactic side effects. The difference of the recombinant polymers to non-anaphylactic T-cell epitope containing allergen derivative is that they contain the IgE-binding sites but have a reduced anaphylactic potential.

Example 2. Mapping the binding site of antibodies in Bet v 1.

Figure 7: Two monoclonal anti-Bet v 1-antibodies (moAb A and B) were used together with three synthetic Bet v 1-derived peptides in ELISA. The sequences of the three peptides are shown in the lower part of the figure and correspond to aa 49-60 (p17), aa 52-63 (p18) and aa 55-66 (p19) of Bet v 1. The peptides were tested for binding to the two Bet v 1 specific monoclonals. The OD values are displayed on the y-axis. Both moAbs bind to the peptides p18 and p19, which are mapped to the first half of Bet v 1.

Table 3. The full table is given at the end of the descriptive part. Monoclonal anti-Bet v 1 antibodies (A,B) inhibit binding of human IgE to recombinant Bet v 1. Dot-blotted Bet v 1 was preincubated with MoAb A and B prior to probing with serum IgE from 60 Bet v 1 allergic individuals. Bound IgE was detected with ^{125}I-labelled anti-human IgE antibodies and quantified by gamma-counting. Inhibition of IgE binding was determined as follows:

$$100-(cpm_1/cpm_2)100 = \%inhibition$$

cpm_1 = count per minutes for incubation with moAb
cpm_2 = count per minutes for incubation buffer
The % inhibition of IgE-binding compared to preincubation with buffer is displayed in the table.

Example 3. Two non-anaphylactic recombinant fragments of Bet v 1

See further Vrtala et al., "Conversion of the major birch pollen allergen, Bet v 1, into two non-anaphylactic T cell epitope containing fragments", J. Clin. Invest. 99(7) April 1997) 1673-1681.

METHODS

Sera from allergic patients, antibodies, protein extracts and E. coli strains. Sera from birch pollen allergic patients and control individuals were characterized by RAST and testing with recombinant allergens as described (Valenta et al., J. Allergy Clin. Immunol. 88 (1991) 889-894; Valenta et al., Int. Arch. Allergy Immunol. 97 (1992) 287-294). In addition, all patients were characterized by case history and skin prick test. The mouse monoclonal antibody moab 14 with specificity for aa 40-65 of Bet v 1 is described (Lebecque et al., J. Allergy Clin. Immunol. 99(3) (1997) 374-384). Natural birch pollen extract was prepared as described (Vrtala et al., Int. Arch. Allergy Immunol. 102 (1993) 160-169). Plasmid pET-17b containing the ampicillin resistance and a T7 promotor was obtained from Novagen, Madison, USA. Recombinant Bet v 1 fragments were expressed in λDE3 lysogens of E. coli strain BL21 (F^- $ompT r_b - m_B -$) (Studier et al., Meth. Enzymol. 185 (1990) 60-89).

Expression of Bet v 1 (aa 1-74, aa 75-160) fragments in E. coli. Recombinant Bet v 1 fragments (aa 1-74, aa 75-160) were generated to maintain the epitopes (aa 40-65) of murine monoclonal antibodies which inhibited binding of allergic patients IgE to Bet v 1 (Lebecque et al., J. Allergy Clin. Immunol. 99(3) (1997) 374-384) and in order to preserve major T-cell epitopes which had been mapped using overlapping peptides synthesized according to the Bet v 1 sequence (Ebner et al., J. Immunol. 150 (1993) 1047-1054). The cDNAs coding for fragment aa 1-74 and aa 75-160 were obtained by PCR amplification of the Bet v 1 cDNA using the following oligonucleotide primers (Pharmacia Biotech AB, Upsala Sweden):

Bet v 1 (aa 1-74)

Sequence Id No 9:

5'GGG AAT TCC ATA TGG GTG TTT TCA ATT AC3'

Sequence Id No 10:

5'CGG *GGT ACC* TTA CTC ATC AAC TCT GTC CTT3'

Bet v 1 (aa 75-160)

Sequence Id No 11:

5'GGG AAT TCC *ATA TGG* TGG ACC ACA CAA ACT3'

Sequence Id No 12:

5'CGG *GGT ACC* TTA GTT GTA GGC ATC GGA3'

The Eco RI sites which were incorporated in the first primers are underlined, Nde I and Kpn I sites are in italics. To improve subcloning efficiency, PCR-products were first cut with Eco RI and Kpn I, purified by preparative agarose gel electrophoresis, subcloned into Eco RI and Kpn I site of plasmid pEt-17b (Novagen, Madison, USA) and transformed into E. coli BL21 (DE3) (Novagen, Madison, USA) by electroporation. Inserts were then excised with Nde I/Kpn I and subcloned again in plasmid pET-17b and transformed. Colonies expressing the correct fragments were identified by immunoscreening using mab 14 for Bet v 1 aa 1-74 and a rabbit anti-Bet v 1 C-terminal antiserum for Bet v1 aa 75-160. DNA from positive clones was isolated using Qiagen tips (Quiagen, Hilden, Germany) and both DNA strands were sequenced according to Sanger using a T7 polymerase sequencing kit (Pharmacia Biotech AB, Uppsala, Sweden) and ^{35}S dCTP (NEN, Stevehage, UK)(24). Recombinant Bet v 1 (aa 1-74 and Bet v1 (aa 75-160) were expressed in E. coli BL21 (DE3) by induction with 0.5 mM IPTG at an OD600 of 0.5-0.8 in liquid culture for 5 hours at 37°C.

Purification of recombinant Bet v1 (aa 1-74) and Bet v1 (aa 75-160). Bet v1 (aa 1-74) and Bet v1 (aa 75-160) were expressed in inclusion bodies isolated as described (Vrtala et al., J. Allergy Clin. Immunol. 97 (1996) 781-787). Inclusion bodies were solubilized with 8M urea, 10 mM Tris, pH 8, 1 mM EDTA (ethylenediaminetetraacetic acid), 5 mm β-mercaptoethanol, diluted with 10 mM Tris, pH 8 to a

concentration of 6 M urea and centrifuged for 15 minutes at 10,000xg to remove insoluble material. The supernatant containing the recombinant protein was dialyzed to a final concentration of 2M urea. Following centrifugation (15min, 10,000xg), the supernatant was applied to a column packed with DEAE (diethylaminoethyl) Sepharose (Pharmacia Biotech AB) and the protein eluted with a 0-0.5M NaCl concentration gradient. Fractions, containing the recombinant protein which was more than 80% pure, were dialyzed against 6M urea, 10mM NaH_2PO_4, pH 4.8 and rechromatographed on a column packed with SP Sepharose (Pharmacia Biotech AB). Fractions containing recombinant Bet v 1 (aa 1-74) or recombinant Bet v 1 (aa75-160) of greater than 95% purity, were dialyzed against 10mM Tris, pH 7.5 and lyophilized until used.

IgE binding capacity of recombinant Bet v 1 and Bet v 1 fragments. Purified recombinant Bet v 1 and Bet v 1 fragments (aa 1-74, aa 75-160) were tested for IgE-binding capacity by Western blotting and in dot blot assays. For immunoblotting, approximately 1 µg/cm purified protein was separated by SDS-PAGE (Fling et al., Anal. Biochem. 155 (1986) 83-88) and blotted onto nitrocellulsoe according to Towbin (Towbin et al., Proc. Natl. Acad. Sci. USA 76 (1979) 4350-4353). To avoid denaturation of the proteins, dot blot experiments were performed in parallel. 1 µg of purified recombinant Bet v 1, 1 µg of each Bet v 1 fragment and 1 µg of bovine serum albumin and human serum albumin (HSA) (negative controls) were dotted on nitrocellulose strips. Nitrocellulose strips containing Western blotted allergens or the dot blotted proteins were incubated with serum IgE from allergic individuals, non-allergic control individuals and buffer without addition of serum as described (Valenta et al., J. Exp. Med. 175 (1992) 377-385). Bound IgE antibodies were detected with ^{125}I labelled anti-human IgE antibodies and visualized by autoradiography.

Results: Sera of birch pollen allergic patients reacted with recombinant Bet v 1 but not with Bet v 1 fragments. Sera of

grass pollen allergic individuals reacted neither with recombinant Bet v 1 nor with the recombinant Bet v 1 fragments.

Circular dichroism showed that the two Bet v 1 fragments showed no tendency to fold, even in the presence of each other.

Histamine release experiments. Granulocytes were isolated from heparinized blood of birch pollen allergic individuals by dextran sedimentation (Valent et al., Proc. Natl. Acad. Sci. USA 86 (1989) 5542-5547). Cells were incubated with different concentrations (0.001µg/ml-10µg/ml) of purified rcombinant Bet v 1, recombinant Bet v 1 fragments (aa 1-74, aa 75-160) separately and in equimolar mixture, or anti-human IgE antibodies. Histamine released in the supernatant was measured by radioimuoassay (RIA) (Immunotech, Marseille, France) (Valenta et al., J. Allergy Clin. Immunol. 91 (1993) 88-97). Total histamine was determined in cell lysates after freeze thawing. Results were obtained as mean values from triplicate determinations and expressed as percentage of total histamine release.

Results: Recombinant Bet v 1 fragments have approximately 1000 fold reduced capacity to induce histamine release from patients basophils compared to recombinant Bet v 1. An equimolar mixture of both Bet v 1 fragments did not induce significant release of histamine compared to each of the tested fragments.

Skin testing. Skin prick tests were performed on the individuals' forearms by placing 20 µl of each solution (Pauli et al., J. Allergy Clin. Immunol. 97 (1996) 1100-1109; Menz et al., Clin. Exp. Allergy 26 (1996) 50-60). Recombinant Bet v 1 and recombinant Bet v 1 fragments were freshly dissolved in a 0.9% w/v sterile sodium chloride solution at concentrations of 100µg/ml and 10µg/ml. As controls birch pollen SQ (standard quality) extract, sodium

chloride solution (negative control) and histamine hydrochloride (positive control)(ALK, Horsholm, Denmark) were used. Each drop was pricked with a fresh prick lancette (ALK, Horsholm, Denmark) and results were recorded after 20 minutes with a ball point pen by transferring the wheal area with a tape paper and by photography. The mean wheal diameter (Dm) was calculated by measuring the maximal longitudinal diameter (D9 and the maximal transversal diameter (d) according to the formula (D+d)/2=Dm.

Results: The two recombinant Bet v 1 fragments, neither alone nor in combination, do not elicit anaphylactic skin reactions compared to the intact recombinant Bet v 1.

Table 1: Proliferation of Bet v 1 specific T-cell clones with recombinant Bet v 1-polymers.

1 Epitop Bet v 1	2 TCC	3 Control	4 Bet v 1	5 Bet v 1-dimer	6 Bet v 1-trimer	7 Bet v 1-tetramer
1-15	CGE 147	15567	47570	97939	67299	79741
1-18	HC 26/II	1264	9977	32667	14170	22178
10-27	WF 110/III	87	6402	12571	5823	9542
10-27	WF 110/III	146	3575	13340	5428	6961
10-27	WF 121/III	287	3914	22099	5117	13000
11-27	TF 7B	359	10492	42352	9869	29900
35-48	HC 3/III	40.7	10499	21301	15761	25609
64-75	CGE 110	612	107103	121178	96135	117930
64-75	CGE 31	2937	71176	55728	38955	67625
64-75	CGE 33	3096	99633	85438	80077	91755
77-93	WF 29R	143	12638	28579	14576	14677
77-93	GZ 17M	172	61463	90586	54988	84237
88-10	CGE 34	515	16045	20531	14176	15217
93-110	TF 1M	438	21423	29741	11500	23454
106-120	WF 9/III	305	43203	81605	32735	65592
109-120	WD 7/III	130	53362	41875	50489	48601
110-128	HC 33/II	134	18099	46022	17917	42051
112-123	WF 112/III	85	10494	12778	7585	11106
112-123	WF 97/III	91	4569	6884	3352	5950
127-138	GZ 10A	182	3347	8379	3227	6645
141-156	TF 10A	215	4862	4438	2232	57
141-156	RR4R	1416	88361	85594	102303	117122
141-156	SAZ 10/IV	612	5121	3830	5207	3979

Table 2: Skin testing with recombinant Bet v 1-monomer and polymers

Individual	Histamine	birch	Bet v 1 monomer 10µg/ml	Bet v 1 monomer 100µg/ml	Bet v 1 dimer 10µg/ml	Bet v 1 dimer 100µg/ml	Bet v 1 trimer 10µg/ml	Bet v 1 trimer 100µg/ml
birch pollen allergic patients								
MS	8	5.5	4	7	3	6	0	0
SF	6	7	8	12	7.5	8	2	5.5
PSt	8	7	6.5	16	6	7	2	4.5
SO	6.5	5.5	5.5	14	0	4.5	0	3.5
SS	4.5	8	5.5	9	0	4	0	0
MD	5.5	9.5	7	11.5	4.5	7	0	5
non-allergic controls								
TB	6	0	0	0	0	0	0	0
VR	8.5	0	0	0	0	0	0	0
CD	6.5	0	0	0	0	0	0	0
TL	9	0	0	0	0	0	0	0

Table 3:

patient #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Inhibition of IgE binding in %	moAb A	49	-	-	57	93	96	-	41	-	41	27	-	29	47	-
	moAb B	96	-	-	45	-	97	-	31	-	45	24	-	-	26	-

patient #		16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Inhibition of IgE binding in %	moAb A	19	21	35	-	36	-	-	10	20	51	30	-	30	-	55
	moAb B	24	25	12	14	21	-	-	-	22	31	33	-	24	-	50

patient #		31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Inhibition of IgE binding in %	moAb A	10	28	8	18	23	23	3	-	46	22	-	8	30	80	33
	moAb B	4	90	5	59	87	97	13	-	18	19	65	80	10	94	17

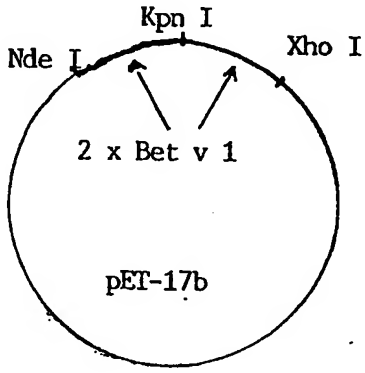
patient #		46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Inhibition of IgE binding in %	moAb A	-	6	54	30	36	12	-	-	-	72	31	1	12	38	-
	moAb B	-	31	97	-	35	8	-	-	-	67	41	-	10	28	-

CONSTRUCTION OF THE BET V 1 POLYMERS

Bet v 1-Dimer

Sequence Id Nos 13 and 14, respectively:

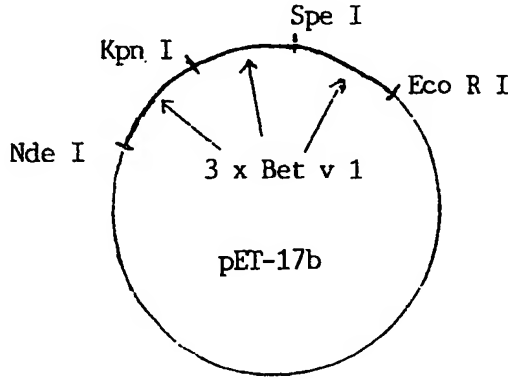
```
ATG.......AAC TTG GTA CCG ATG.....AAC TAA
Met        Asn Leu Val Pro Met      Asn End
   Bet v 1                   Bet V 1
```



Bet v 1-Trimer

Sequence Id Nos 15 and 16, respectively:

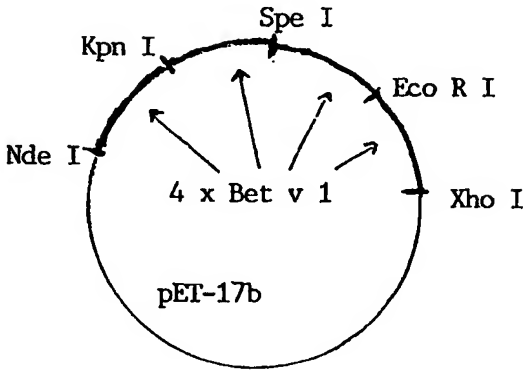
```
ATG.....AAC TTG GTA CCG ATG.....AAC CCA CTA GTA ATG.....AAC
Met.....Asn Leu Val Pro Met.....Asn Pro Leu Val Met.....Asn
  Bet v 1                 Bet v 1                   Bet v 1

GGA TTC TGC AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAG ATC
Glu Phe Cys Arg Tyr Pro Ser His Trp Arg Pro Leu Glu Gln Ile

CGG CTG CTA ACA AAG CCC GAA AGG AGG CTG AGT TGG CTG CTG CCA
Arg Leu Leu Thr Lys Pro Glu Arg Lys Leu Ser Trp Leu Leu Pro

CCG CTG AGC AAT AAC TAG
Pro Leu Ser Asn Asn End
```



Bet v 1-tetramer
Sequence Id Nos 17 and 18, respectively:

ATG.....AAC TTG GTA CCG ATG.....AAC CCA CTA GTA ATG.....AAC
Met.....Asn Leu Val Pro Met.....Asn Pro Leu Val Met.....Asn
Bet v 1 Bet V 1 Bet v 1

GAA TTC ATG.....AAC TAA
Glu Phe Met.....Asn End
Bet v 1



Claims

1. An immunogen derived from a protein allergen, **characterized** in that said immunogen comprises:

a) a polymeric form of a non-anaphylactic immunogenic recombinant fragment of the protein allergen, said fragment containing an IgG epitope partly but not wholly overlapping an IgE epitope of the protein allergen, in which polymeric form said fragment constitutes the monomeric units; or

b) a recombinant polymeric form of said protein allergen in which the protein allergen constitutes the monomeric units.

2. The immunogen according to claim 1, **characterized** in that the polymeric form of said fragment is recombinantly produced.

3. The immunogen according to claim 1 or 2, **characterized** in that said monomeric units are separated from each other by an oligopeptide linker, typically consisting of a 1-30 amino acid residue that may be hydrophilic.

4. The immunogen according to any one of claims 1 to 3, **characterized** in that said immunogen also contains a carrier for said polymeric forms in (a) and (b), respectively.

5. The immunogen according to any one of claims 1 to 4, **characterized** in that the protein allergen is Bet v 1.

6. The immunogen according to any one of claims 1 to 5, **characterized** in that the number of the monomeric units is an integer selected from 2-10.

7. The use of the immunogen according to any one of claims 1 to 6 for in vitro diagnosis of type I allergy in a mammalian individual.

8. The use according to claim 7, **characterized** in that the number of monomeric units is an integer selected from 2-10.

9. The use of the immunogen according any one of claims 1 to 6 for the preparation of a medicament to be used in the hyposensitization of a mammalian individual suffering from a type I allergy, or for the preparation of a reagent to be used in diagnosis in vivo of type I allergy.

10. The use according to claim 9, **characterized** in that the number of monomeric units is an integer selected from 2-10.

11. A method for the hyposensitization of a mammal suffering from IgE mediated allergy against a protein allergen, which method comprises the step of presenting the immune system of the mammal in vivo to an effective amount of an immunogen hyposensitizing the mammal against the allergen, **characterized** in that the immunogen comprises:

a) a polymeric form of a non-anaphylactic immunogenic recombinant fragment of the protein allergen, said fragment containing an epitope partly but not wholly overlapping an IgE epitope of the protein allergen, in which polymeric form said fragment constitutes the monomeric units; or

b) a recombinant polymeric form of said protein allergen in which the protein allergen constitutes the monomeric units.

12. The method according to claim 11, **characterized** in that the immunogen is a polymeric form of said fragment and is recombinantly produced.

13. The method according to claim 11 or 12, **characterized** in that said monomeric units are separated from each other by an oligopeptide linker, typically consisting of a 1-30 amino acid residue that may be hydrophilic.

14. The method according to any one of claims 11 to 13, **characterized** in that said immunogen also contains a carrier for the polymeric forms in (a) and (b), respectively.

15. The method according to any one of claims 11 to 14, **characterized** in that the protein allergen is Bet v 1.

16. The method according to any one of claims 11 to 15, **characterized** in that the number of monomeric units is an integer selected from 2-10.

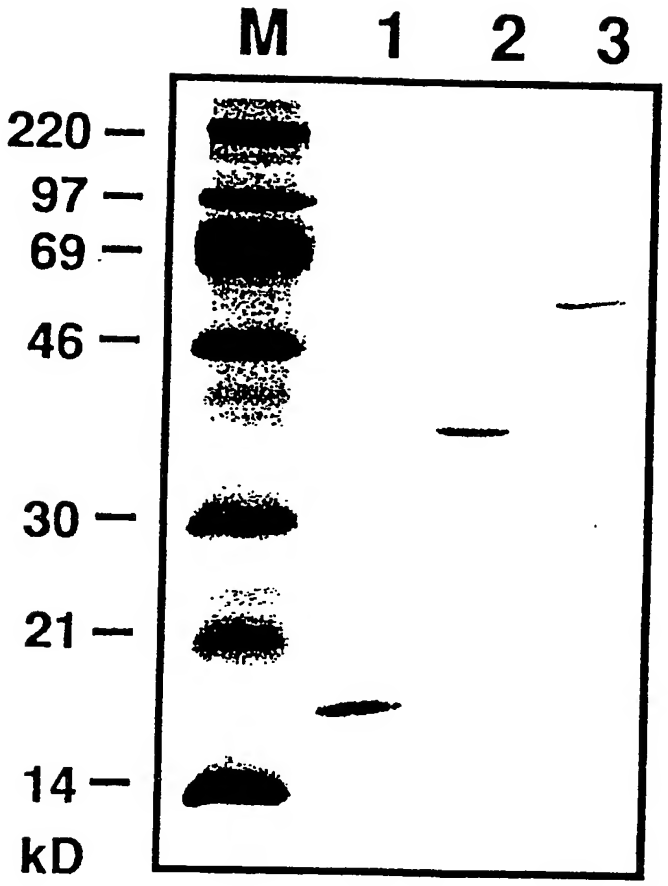


Fig. 1

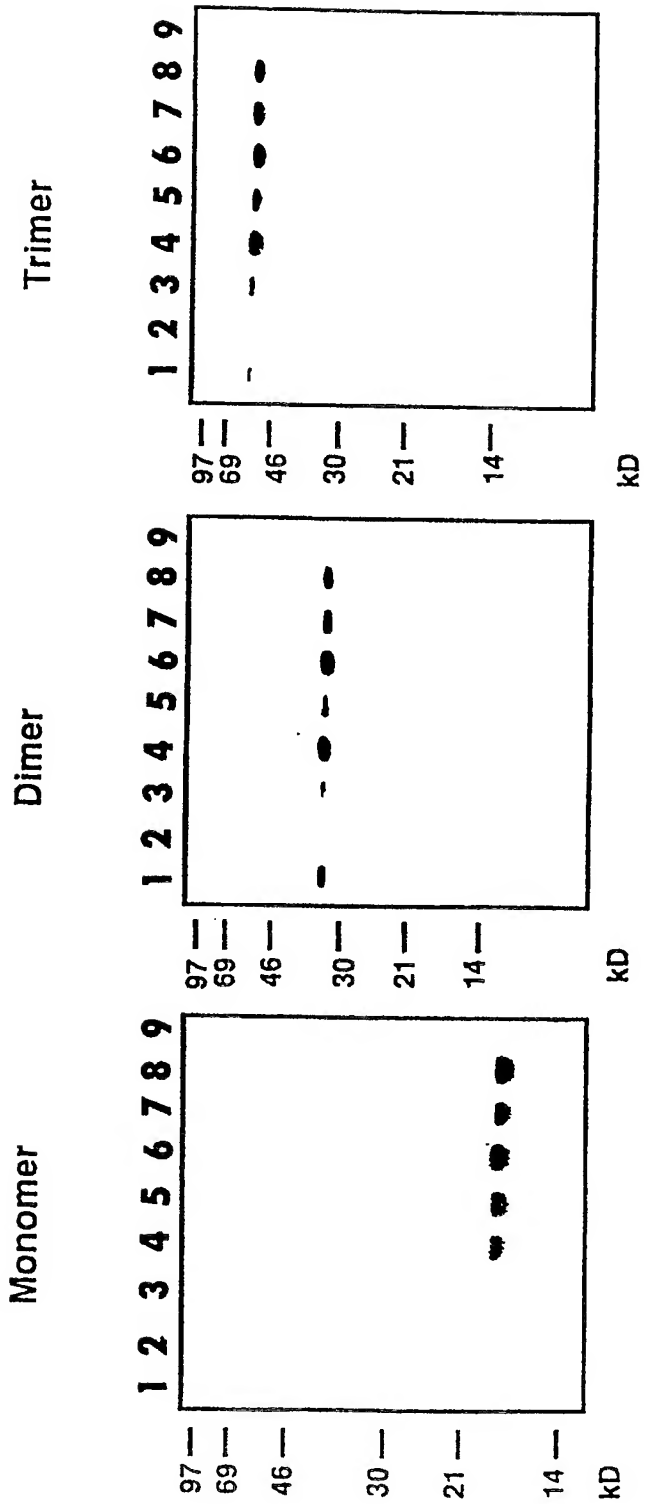


Fig. 2

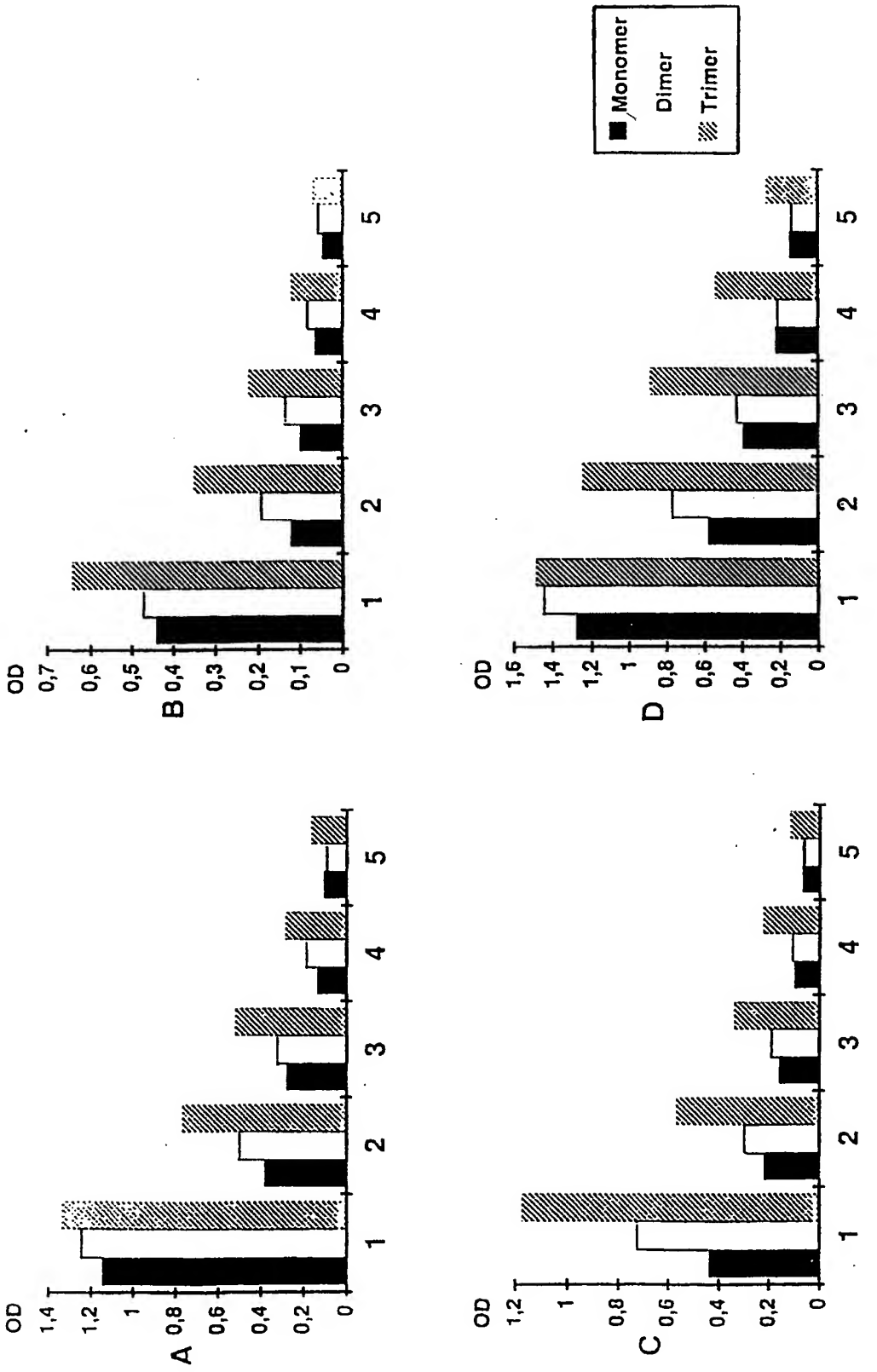


Fig. 3

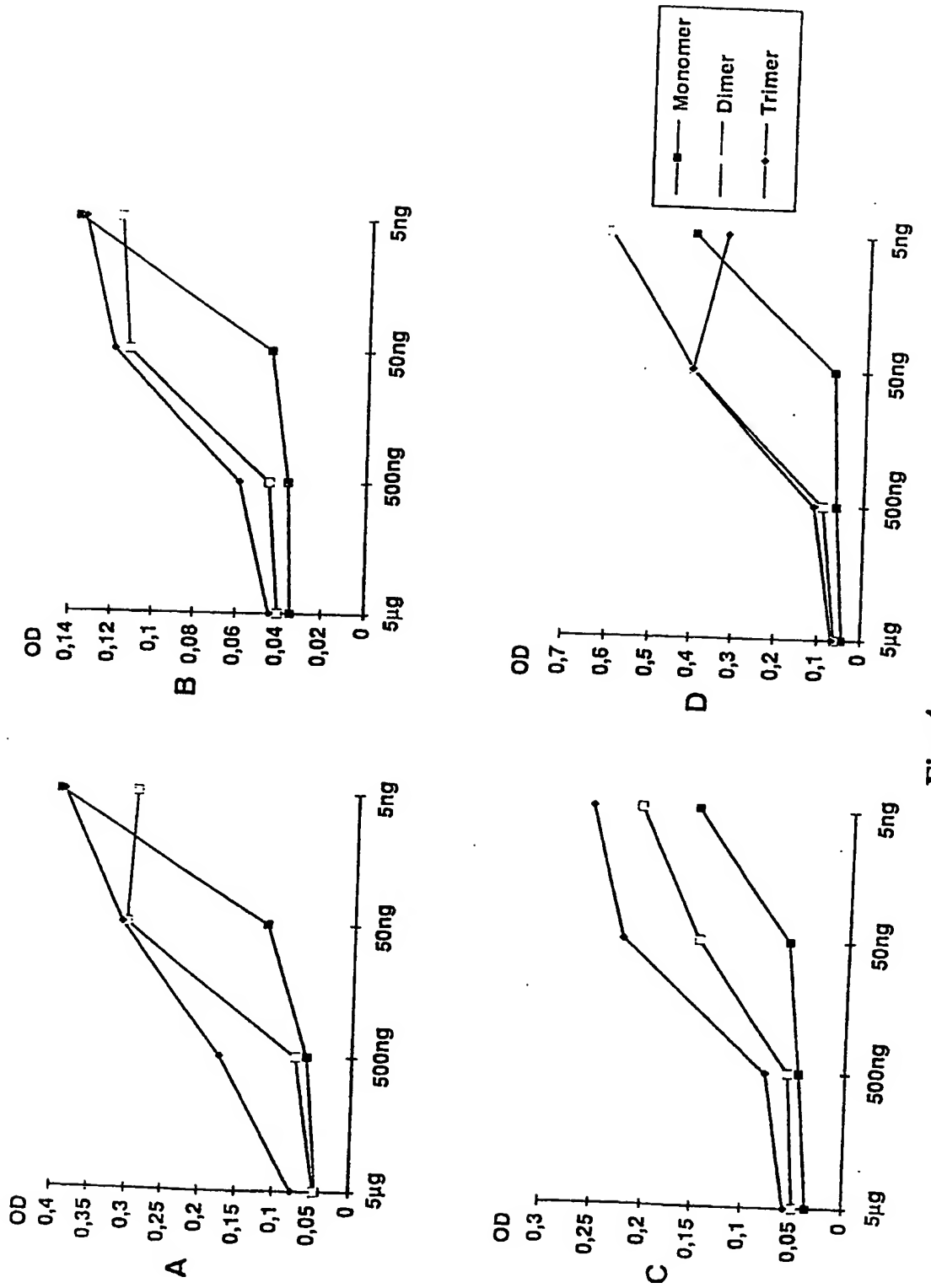
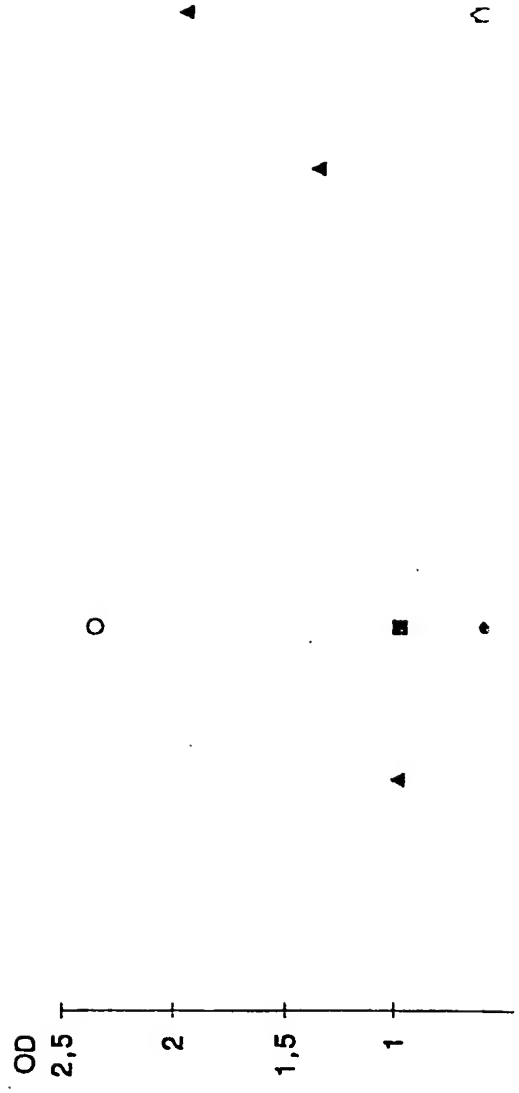


Fig. 4


OD
2,5
2
1,5
1

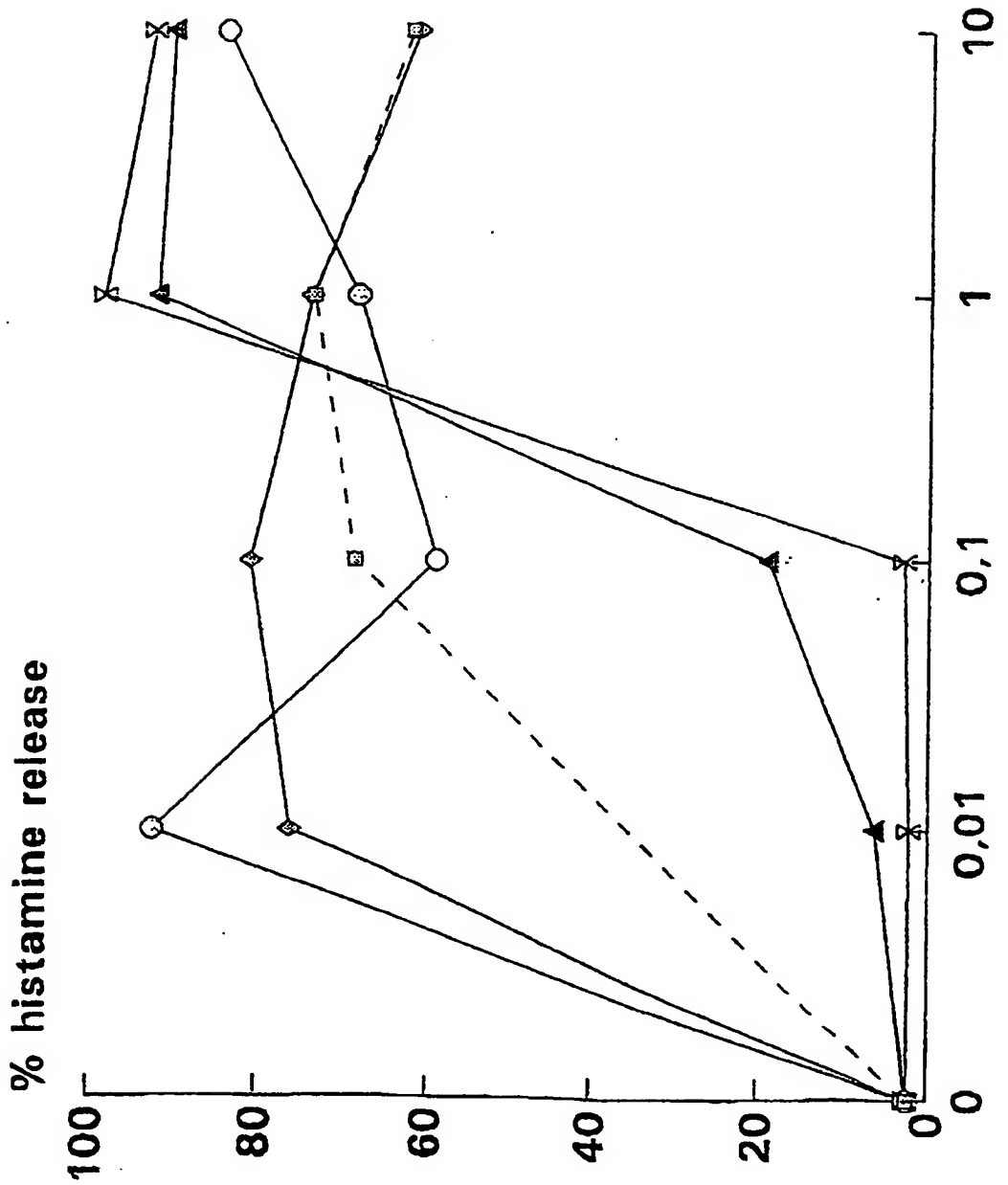
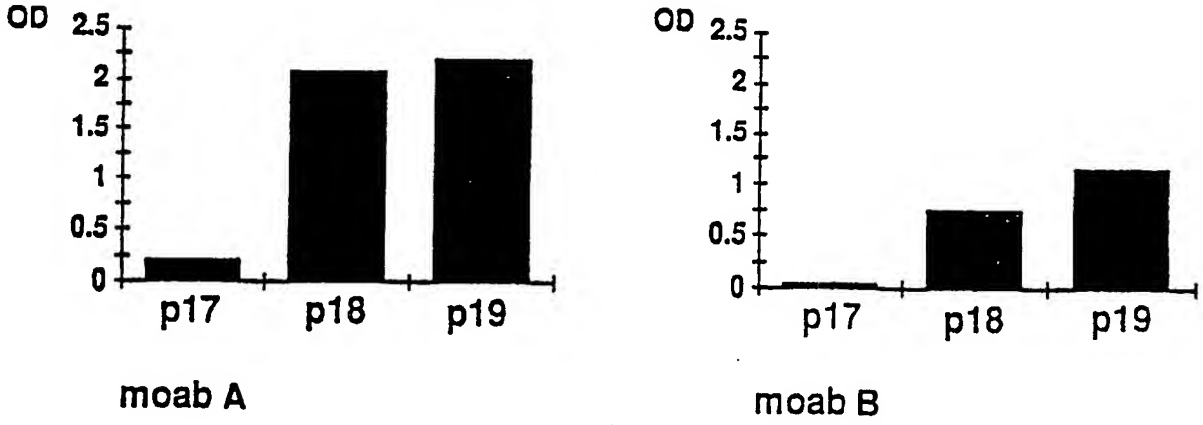

anti IgE
Bet v 1
Dimer
Trimer
Tetramer
% histamine release
100
80
60
40
20
0
0
0,01
0,1
1
10

Fig. 6



p17	GPGTIKKISFPE	aa 49-60
p18	TIKKISFPEGFP	aa 52-63
p19	KISFPEGFPFKY	aa 55-66

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01765

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/35, C07K 14/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of clinical investigation, Volume 99, No 7, April 1997, Susanne Vrtalaet al, "Conversion of the Major Birch Pollen Allergen, Bet v 1, into Two Nonanaphylactic T Cell Epitope-containing Fragments", page 1673 - page 1681, see Table 1 and page 1679 - 1680	
Y		1-10
	--	
A	WO 9603106 A2 (UNIVERSITY OF MANITOBA), 8 February 1996 (08.02.96), see Table 1, examples 2 and 3, pages 7-9, 12-15, page 24, lines 33-36	1,7,9
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 1999	29 -01- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01765

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9410194 A2 (BIOMAY PRODUKTIONS- UND HANDELSGESELLSCHAFT M.B.H.), 11 May 1994 (11.05.94), see pages 5-6	5
A	WO 9534578 A1 (PHARMACIA AB), 21 December 1995 (21.12.95)	1
A	US 5449669 A (DEAN D. METCALFE ET AL), 12 Sept 1995 (12.09.95), see column 11, line 28 - column 12	1
Y	Dialog Information Service, file 154, MEDLINE, Dialog accession no. 08362396, Medline accession no. 98055718, Tamborini E et al: "Biochemical and immunological characterization of recombinant allergen Lol p 1"; Eur J Biochem, Nov 1 1997, 249 (3)p886-94	1-10
A	Dialog Information Services, file 154, MEDLINE, Dialog accession no. 08635889, Medline accession no. 96245983, Zhang L et al: "Multiple B- and T-cell epitopes on a major allergen of Kentucky Bluegrass pollen"; & Immunology, Feb 1996, 87 (2) p283-90	1,7,9
Y	WO 9724139 A1 (UNIVERSITY OF ARKANSAS), 7 October 1997 (07.10.97), see pages 73-74, pages 125-129, pages 149-150 and in particular page 127, lines 7-13	1-10
Y	WO 9211029 A1 (THE JOHNS HOPKINS UNIVERSITY), 9 July 1992 (09.07.92), see claim 1 and examples 5 and 12 and pages 71-73	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

21/12/98

International application No.
PCT/SE 98/01765

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9603106 A2	08/02/96	AU 3338195 A	22/02/96
WO 9410194 A2	11/05/94	AU 5411594 A	24/05/94
WO 9534578 A1	21/12/95	AU 2759895 A	05/01/96
		EP 0763059 A	19/03/97
		JP 10501417 T	10/02/98
		SE 9402089 D	00/00/00
US 5449669 A	12/09/95	NONE	
WO 9724139 A1	07/10/97	AU 7243396 A	28/07/97
		EP 0873135 A	28/10/98
WO 9211029 A1	09/07/92	CA 2098281 A	18/06/92
		EP 0572443 A	08/12/93
		JP 6503833 T	28/04/94